ENGINEERED PHENYLALANINE AMMONIA LYASE POLYPEPTIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to US Prov. Pat. Appln. Ser. No. 61/813,586 filed April 18, 2013, and US Prov. Pat. Appln. Ser. No. 61/897,932, filed October 31, 2013, both of which are incorporated by reference in their entireties for all purposes.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

[0002] The Sequence Listing written in file CX7-131WO2_ST25.TXT, created on April 15, 2014, 127,412 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference.

FIELD OF THE INVENTION

[0003] The present invention provides engineered phenylalanine ammonia-lyase (PAL) polypeptides and compositions thereof, as well as polynucleotides encoding the engineered phenylalanine ammonia-lyase (PAL) polypeptides. In some embodiments, the engineered PAL polypeptides are optimized to provide enhanced catalytic activity, as well as reduced sensitivity to proteolysis and increased tolerance to acidic pH levels. In some embodiments the engineered PAL polypeptides are deimmunized. The invention also relates to the use of the compositions comprising the engineered PAL polypeptides for therapeutic and industrial purposes.

BACKGROUND OF THE INVENTION

[0004] Phenylalanine ammonia-lyase (PAL) along with histidine ammonia-lyase (HAL) and tyrosine ammonia-lyase (TAL) are members of the aromatic amino acid lyase family (EC 4.3.1.23-1.25 and 4.3.1.3). More specifically the enzymes having PAL activity (EC 4.3.1.23-1.25 and previously classified as EC4.3.1.5) catalyze the nonoxidative deamination of L-phenylalanine into (E)-cinnamic acid. PAL is a non-mammalian enzyme that is widely distributed in plants and has also been identified in fungi and a limited number of bacteria. PAL enzymes may be used as a therapeutic protein for the treatment of the metabolic disorder, phenylketonuria (PKU). PKU is an autosomal metabolic genetic disorder in which the hepatic enzyme phenylalanine hydroxylase (PAH) or one or more of the enzymes involved in the synthesis or recycling of the co-factor tetrahydrobiopterin, is nonfunctional due to a mutation in one of the corresponding genes. This lack of functionality results in high levels of phenylalanine in the bloodstream. The phenylalanine is converted into phenylpyruvate (phenylketone) and other derivatives. In humans, if PKU is not treated early, high levels of phenylalanine and some

of its breakdown products can cause significant medical problems including intellectual disability, microcephaly and seizures. Numerous studies have focused on the use of PAL in the treatment of PKU by enzyme substitution (Ambrus et al., Science 201:837–839 [1978]; Bourget et al., Appl. Biochem. Biotechnol., 10:57–59 [1984]; and Sarkissian et al., Proc. Natl. Acad. Sci. USA 96:2339–2344 [1999]).

[0005] One method of detoxifying phenylalanine in the blood stream is the use of injectable recombinant PAL and PAL variants modified by pegylation (PEG-PAL). Pegylation has been shown to improve enzyme half-life and reduce subject antigenic response (See e.g., WO 2008/153776, WO 2011/097335, and US Pat. No. 7,531,341). PAL variants useful in PEG-PAL compositions have been described as variants of wild-type *Nostoc punctiforme* (NpPAL); *Anabaena variabilis* (AvPAL) and *Rhodosporidium toruloides* (RtPAL). In particular, variants of wild-type AvPAL have been described wherein the cysteine residues at positions 64, 318, 503 and 565 have been substituted with serine (See e.g., US Pat Nos: 7,790,433; 7,560,263; and 7,537,923).

[0006] An alternative route of PAL administration as a means of reducing plasma concentration of L-phenylalanine in PKU subjects is a non-invasive formulation such as an oral formulation (Sarkissian et al., Proc. Natl. Acad. Sci. USA 96:2339–2344 [1999]). A key advantage of oral delivery of PAL is the reduced exposure of the enzyme to the immune system thereby minimizing the immune response which is observed with injectable PEG-PAL. However, a major limitation for the oral formulation of PAL is loss of enzyme activity in the stomach and intestinal lumen. In order to be effective and functional PAL must resist degradation by acidic pHs and proteases such as trypsin, chymotrypsin, carboxypeptidases and pepsin that normally degrade proteinaceous foods to oligopeptides and amino acids. In some previous studies (Sarkissian, *supra*) in order to achieve a significant effect for the oral administration of PAL, a large amount of the enzyme was required partly due to enzymatic degradation by proteases and partly due to relatively low specific activity at pH 7.0. Various means have been explored to suppress PAL degradation upon digestion (Kim et al., Molec. Therap., 10:220 – 224 [2004]; and Shah et al., Int. J. Pharmaceut., 356:61–68 [2008]).

[0007] One approach to increase the effectiveness of PAL under the harsh conditions of the digestive tract is to provide engineered PAL polypeptides that are tolerant to the inherent harsh conditions. Kang et al. used site directed mutagenesis of a chymotrypsin cleavage site and pegylation of surface lysines of an AvPAL to reduce proteolytic inactivation (See, Kang et al., Mol. Gen. Metabol., 99:4-9 [2010]). In these studies ten cleavage sites were specifically mutated and all but two of these resulting mutants (F18A and R94G) lost more than 50% of the original enzyme activity. None of the mutants showed increased activity and the F18A mutant showed a slight increase in trypsin resistance (Kang et al., *supra*). Further studies with PAL, while effective, generally have not resulted in a longer lived enzyme. Therefore, oral administration of previously described PAL mutants and derivatives thereof does not result in effective treatment of PKU.

[0008] Despite the progress made with various formulations of PAL there remains a need for PAL polypeptides having improved properties for oral administration. These improved properties include without limitation a greater half-life, increased catalytic activity, improved stability to the conditions in the digestive track and reduced aggregation.

[0009] In addition to therapeutic applications PAL enzymes may also be used in the industrial synthesis of L-phenylalanine and other substituted L-phenylalanine derivatives. These derivatives may then be used as pharmaceutical precursors (Gloge et al., Chem., 6: 3386-3390 [2000]; Bartsch et al., Prot. Eng. Des. Sel., 23:929–933 [2010]; and Turner, Curr. Opin. Chem. Biol., 234-240 [2011]).

[0010] PAL enzymes may also be used in agricultural applications. PAL plays a significant role in biosynthesis of phenylpropanoids (such as flavonoids and lignin) in plants, fungi and bacteria and can act as a defense related enzyme (Bate et al., Proc. Natl. Acad. Sci. USA 91:7608-7612 [1994]). Modulation of PAL activity by using recombinant polypeptides having PAL activity could potentially lead to effective herbicides.

SUMMARY OF THE INVENTION

[0011] The present invention provides engineered phenylalanine ammonia-lyase (PAL) polypeptides and compositions thereof, as well as polynucleotides encoding the engineered phenylalanine ammonia-lyase (PAL) polypeptides. In some embodiments, the engineered PAL polypeptides are optimized to provide enhanced catalytic activity, as well as reduced sensitivity to proteolysis and increased tolerance to acidic pH levels. In some embodiments the engineered PAL polypeptides are deimmunized. The invention also relates to the use of the compositions comprising the engineered PAL polypeptides for therapeutic and industrial purposes. In some embodiments, the present invention is directed to engineered phenylalanine ammonia-lyase (PAL) polypeptides and biologically active fragments and analogs thereof having improved properties such an increased tolerance to acidic pH and/or reduced sensitivity to proteolysis.

[0012] The present invention is directed to engineered PAL polypeptides and biologically active fragments and analogs thereof having improved properties when compared to a wild-type PAL enzyme or a reference PAL polypeptide under essentially the same conditions. The invention is further directed to methods of using the engineered PAL polypeptides and biologically active fragments and analogs thereof in therapeutic and/or industrial compositions and methods of using such compositions for therapeutic and/or industrial purposes.

[0013] In a first aspect, the invention provides engineered phenylalanine ammonia-lyase (PAL) polypeptides wherein the engineered PAL polypeptide has an improved property selected from the group of i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, or a combination of any of i), ii), iii) or iv) as compared to the reference sequence when measured under essentially the same conditions. In some specific embodiments, the engineered PAL polypeptides have two improved properties. In other specific

embodiments, the improved property is reduced sensitivity to proteolysis and in yet in other specific embodiments, the improved property is increased tolerance to acidic pH.

[0014] In a second aspect, the engineered PAL polypeptides include proteins comprising at least 85% amino acid sequence identity to SEQ ID NO:4, or a functional fragment thereof and an amino acid residue difference at a position corresponding to positions X39; X91; X158; X180; X195; X243; X245; X256; X257; X270; X290; X307; X308; X326; X349; X364; X394; X399; X400; X404; X407; X443; X453; X459; X460; X463; X474; X522; X524; and X528, when optimally aligned with the polypeptide of SEQ ID NO:4.

[0015] In some specific embodiments of the first and second aspects, the engineered PAL polypeptides comprise at least an amino acid residue difference of one or more amino acid residue positions corresponding to A39; A91; Y158; S180; K195; T243; I245; A256; L257; N270; N290; H307; E308; I326; L349; L364; A394; S399; N400; P404; L407; F443; N453; Y459; T460; T463; N474; K522; T524; and P528, when optimally aligned with the polypeptide of SEQ ID NO:4. In some specific embodiments, the engineered PAL polypeptides comprise at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, and at least 20 amino acid residue differences from the reference polypeptide comprising the amino acid sequence of SEQ ID NO:4.

[0016] In other specific embodiments of the first and second aspects, the engineered PAL polypeptides comprise at least 90%, (at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99%) amino acid sequence identity to SEQ ID NO:4. In yet further specific embodiments, the engineered PAL polypeptides comprise at least 90%, (at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99%) amino acid sequence identity to SEQ ID NO:4, and comprise one or more of the following substitutions A39V; A91V; Y158H; S180A; K195E; T243I/L; I245L; A256G; L257W/A; N270K; N290G; H307G/Q/M; E308Q; I326F; L349M; L364Q; A394V; S399N; N400K; P404A; L407V; F443H; N453G; Y459F; T460G; T463N; N474Q; K522Y/F/N; T524S; and P528L.

[0017] In other specific embodiments, the engineered PAL polypeptides are derived from a wild-type *Anabaena variabilis* PAL (such as ATCC29413; NCBI protein reference sequence YP_324488.1; SEQ ID NO:4).

[0018] In a third aspect, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity encompassed by the invention comprises an amino acid sequence having at least 99% sequence identity to SEQ ID NO:10, or a functional fragment thereof.

[0019] In a fourth aspect, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity encompassed by the invention comprise an amino acid sequence having at least 95% sequence identity to SEQ ID NO:10, or a functional fragment thereof and further comprising an amino acid residue difference as compared to SEQ ID NO:10, at one, two, three, four, five, or six more amino acid positions.

[0020] In a fifth aspect, the invention provides a polynucleotide sequence encoding any one of the engineered PAL polypeptides as described herein.

[0021] In a sixth aspect, the invention provides a pharmaceutical composition or an industrial composition comprising any one of the engineered PAL polypeptides as described herein.

[0022] In some embodiments, the present invention provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:4 or a functional fragment thereof; b) an amino acid residue difference as compared to SEQ ID NO:4 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation or a combination of any of i), ii), iii) or iv) as compared to the reference sequence. In some embodiments, one or more amino acid positions are selected from X39; X54; X59; X73; X91; X158; X112, X134, X180; X195; X240; X243; X245; X256; X257; X270; X290; X304, X305; X307; X308; X326; X349; X353; X364; X394; X399; X400; X404; X407; X443; X453; X459; X460; X463; X474; X509; X521; X522; X524; X528; X546; X564; and/or combinations thereof when optimally aligned with the amino acid sequence of SEQ ID NO: 4. In some additional embodiments, the improved property is selected from reduced sensitivity to proteolysis and/or increased tolerance to acidic pH. In yet additional embodiments, the reference sequence is a wild-type PAL derived from *Anabaena variabilis*. In some further embodiments, the amino acid residue of the reference sequence of SEQ ID NO:4 corresponds to A39; T54; G59, S73; A91; Y158; S180; K195; A112; R134; Q240; T243; I245; A256; L257; N270; N290; Y304; R305; H307; E308; I326; L349; D353; L364; A394; S399; N400; P404; L407; F443; N453; Y459; T460; T463; N474; E509; Q521; K522; T524; P528; S546; and/or P564. In some embodiments, the amino acid residue difference as compared to SEQ ID NO:4 is selected from one or more of the following substitutions A39V; T54K; G59R; S73K; A112C; R134Q; A91V; Y158H; S180A; K195E; Q240R/W; T243I/L; I245L; A256G; L257W/A; N270K; N290G; Y304H; R305M; H307G/Q/M; E308Q; I326F; L349M; D353A/N; L364Q; A394V; S399N; N400K; P404A; L407V; F443H; N453G; Y459F; T460G; T463N; N474Q; E509L; Q521K/S; K522Y/F/N; T524S;P528L; S546R; and P564 G/L/M; when optimally aligned with the polypeptide of SEQ ID NO:4. In some further embodiments, the engineered polypeptide has at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% sequence identity to reference sequence SEQ ID NO:4. In some further embodiments, the engineered polypeptide has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to reference sequence SEQ ID NO:4. In some additional embodiments, the engineered polypeptide has at least about 90% sequence identity to reference sequence SEQ ID NO:4. In some additional embodiments, the engineered polypeptide has at least about 95% sequence identity to reference sequence SEQ ID

NO:4. In some further embodiments, the engineered polypeptide has at least about 90% sequence identity to SEQ ID NO:4; and an amino acid residue difference at position H307. In some further embodiments, the engineered polypeptide has at least 90% sequence identity to reference sequence SEQ ID NO:4. In some additional embodiments, the engineered polypeptide has at least 95% sequence identity to reference sequence SEQ ID NO:4. In some further embodiments, the engineered polypeptide has at least 90% sequence identity to SEQ ID NO:4; and an amino acid residue difference at position H307. In some additional embodiments, the amino acid residue difference is H307G/Q/M. In some further embodiments, the amino acid residue difference is selected from a combination of one or more of A39; A91; Q240; A256; N290; Y304; R305; H307; D353 A394; S399; P404; L407; Q521; K522; and T524.

[0023] The present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising an amino acid sequence having at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about100% sequence identity to SEQ ID NO:6, 8, 10, 12, and/or 14, or a functional fragment thereof. In some embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprise an amino acid sequence having at least about 95% sequence identity to SEQ ID NO:6, 8, 10, 12, and/or 14, or a functional fragment thereof.

[0024] In some further embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprise an amino acid sequence having at least about 99% sequence identity to SEQ ID NO:6, 8, 10, 12, and/or 14, or a functional fragment thereof.

[0025] The present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising an amino acid sequence having at least about at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at 100% sequence identity to SEQ ID NO:6, 8, 10, 12, and/or 14, or a functional fragment thereof. In some embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprise an amino acid sequence having at least about 95% sequence identity to SEQ ID NO:6, 8, 10, 12, and/or 14, or a functional fragment thereof. In some further embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprise an amino acid sequence having at least about 99% sequence identity to SEQ ID NO:6, 8, 10, 12, and/or 14, or a functional fragment thereof. In some embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprise an amino acid sequence having at least 95% sequence identity to SEQ ID NO:6, 8, 10, 12, and/or 14, or a functional fragment thereof. In some further embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprise an amino acid sequence having at least 99% sequence identity to SEQ ID NO:6, 8, 10, 12, and/or 14, or a functional fragment thereof.

[0026] The present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising an amino acid sequence having at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about100% sequence identity to SEQ ID NO:4, or a functional fragment thereof, wherein the engineered polypeptide is deimmunized. The present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO:4, or a functional fragment thereof, wherein the engineered polypeptide is deimmunized. In some embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprise an amino acid sequence having at least 95% sequence identity to SEQ ID NO:4, or a functional fragment thereof, wherein the engineered polypeptide is deimmunized. In some additional embodiments, the engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity is a variant PAL provided in any of Tables 9-1 through 9-7. In some embodiments, the deimmunized engineered having phenylalanine ammonia-lyase (PAL) activity comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO:6, 8, 10, 12, and/or 14.

[0027] The present invention also provides polynucleotide sequences encoding the engineered polypeptides having PAL activity provided herein. In some embodiments, the polynucleotide sequence is operably linked to a control sequence. The present invention further provides vectors comprising at least one polynucleotide sequence encoding at least on engineered polypeptide having PAL activity. The present invention also provides host cells transformed with at least one polynucleotide sequence encoding an engineered polypeptide having PAL activity, as provided herein.

[0028] The present invention further provides methods of producing an engineered PAL polypeptide in a host cell, comprising culturing a host cell comprising at least one polynucleotide encoding at least one engineered PAL polypeptide under suitable culture conditions. The present invention further provides methods of producing an engineered PAL polypeptide in a host cell, comprising culturing a host cell comprising a polynucleotide encoding the engineered PAL polypeptide under suitable culture conditions. In some embodiments, the methods further comprise recovering the engineered PAL polypeptide from the culture and/or host cells.

[0029] The present invention also provides compositions comprising at least one engineered polypeptide having PAL activity, as provided herein. In some embodiments, the composition is a pharmaceutical composition further comprising a pharmaceutically acceptable carrier. The present invention further provides uses of these compositions.

[0030] The present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least about 85%, at least about

86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or greater sequence identity to a reference sequence having phenylalanine ammonia-lyase (PAL) activity, or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to a reference sequence having phenylalanine ammonia-lyase (PAL) activity, or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence having phenylalanine ammonia-lyase (PAL) activity. In some embodiments, the reference sequence is a prokaryotic PAL, while in some other embodiments, the reference sequence is a eukaryotic PAL. In some embodiments, the reference sequence is a bacterial PAL (e.g., *Anabaena variabilis* PAL), while in some other embodiments it is a human or other PAL. In some further embodiments, the reference sequence is a wild-type sequence (e.g., wild-type *A. variabilis* PAL), while in some alternative embodiments, the reference sequence is a variant enzyme (e.g., an engineered polypeptide having PAL activity).

[0031] In some embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity of the present invention comprise: a) an amino acid sequence having at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or greater sequence identity to reference sequence SEQ ID NO:4 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:4, or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:4.

[0032] In some additional embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprise: a) an amino acid sequence having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater sequence identity to reference sequence SEQ ID NO:4 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:4 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:4.

[0033] In some embodiments, the engineered polypeptides having phenylalanine ammonia lyase activity (PAL) comprise at least one substitution(s) at one or more of the following amino acid positions: 20, 24, 27, 39, 43, 45, 47, 54, 58, 59, 62, 70, 73, 80, 82, 91, 94, 98, 104, 105, 110, 112, 115, 117, 118, 119, 121, 123, 124, 125, 126, 127, 128, 129, 130, 131, 133, 134, 135, 139, 140, 141, 142, 143, 144, 145, 146, 147, 149, 150, 151, 153, 154, 156, 157, 158, 159, 172, 174, 175, 176, 177, 178, 180, 187, 191, 195, 199, 205, 206, 210, 212, 213, 214, 232, 240, 243, 245, 247, 248, 250, 256, 257, 266, 270, 275, 278, 279, 285, 286, 289, 290, 292, 304, 305, 307, 308, 309, 319, 321, 326, 331, 332, 334, 349, 353, 355, 364, 365, 369, 370, 371, 372, 374, 375, 377, 378, 379, 381, 382, 383, 384, 385, 387, 389, 394, 396, 399, 400, 403, 404, 407, 417, 418, 425, 431, 432, 433, 434, 435, 436, 437, 438, 439, 443, 446, 447, 453, 456, 459, 460, 461, 463, 471, 472, 473, 474, 475, 476, 477, 478, 479, 482, 483, 503, 507, 509, 521, 522, 524, 525, 528, 538, 546, 547, 551, 558, 560, 564, 565, and/or any combinations thereof, wherein the amino acid positions are numbered with reference to SEQ ID NO:4. In some embodiments, the amino acid residue of the reference sequence of SEQ ID NO:4 corresponds to A39, T54, G59, S73, A91, Y158, S180, K195, A112, R134, Q240, T243, I245, A256, L257, N270, N290, Y304, R305, H307, E308, I326, L349, D353, L364, A394, S399, N400, P404, L407, F443, N453, Y459, T460, T463, N474, E509, Q521, K522, T524, P528, S546, and/or P564. In some additional embodiments, the amino acid residue difference as compared to SEQ ID NO:4 is selected from one or more of the following substitutions A39V, T54K, G59R, S73K, A112C, R134Q, A91V, Y158H, S180A, K195E, Q240R/W, T243I/L, I245L, A256G, L257W/A, N270K, N290G, Y304H, R305M, H307G/Q/M, E308Q, I326F, L349M, D353A/N, L364Q, A394V, S399N, N400K, P404A, L407V, F443H, N453G, Y459F, T460G, T463N, N474Q, E509L, Q521K/S, K522Y/F/N, T524S,P528L, S546R, and P564 G/L/M, when optimally aligned with the polypeptide of SEQ ID NO:4. In some further embodiments, the engineered polypeptide has at least about 90% sequence identity to SEQ ID NO:4; and an amino acid residue difference at position H307. In some embodiments, the amino acid residue difference is H307G/Q/M. In some still further embodiments, the amino acid residue difference is selected from a combination of one or more of A39, A91, Q240, A256, N290, Y304, R305, H307, D353, A394, S399, P404, L407, Q521, K522, and T524. In some additional embodiments, the improved property of the engineered polypeptides having phenylalanine ammonia lyase activity (PAL) is selected from reduced sensitivity to proteolysis and/or increased tolerance to acidic pH.

[0034] The present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or greater sequence identity to reference sequence SEQ ID NO:10 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:10 or the functional

fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:10.

[0035] In some embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater sequence identity to reference sequence SEQ ID NO:10 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:10 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:10.

[0036] In some embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:10 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:10 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:10.

[0037] In some embodiments, the present invention also provide engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:10 and at least one amino acid residue difference as compared to SEQ ID NO:10 and that exhibits at least one improved property selected from enhanced catalytic activity, reduced sensitivity to proteolysis, increased tolerance to acidic pH, reduced aggregation, and/or reduced immunogenicity, as compared to the SEQ ID NO:10.

[0038] In some embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the amino acid residue difference as compared to SEQ ID NO:10, is selected from one or more of the following substitutions or substitution sets:
I27E/V39A; I27E/V39A/R43L/V105C/A153R/L214E/P266H/L278D/C503Q;
I27E/V39A/R43L/L214E/A547D;
I27E/V39A/V105C/A112C/R134Q/L214E/L278D/C503Q/A547D/C565N;
I27E/V39A/V105C/A112C/R134Q/A153R/Q205T/L214E/P266H/L278D/C503Q/A551D;

I27E/V39A/V105C/A112C/Q205T/P210C/P266H/C503Q/A547D;
I27E/V39A/V105C/A112C/Q205T/P266H/I285E/C503Q/A551D;
I27E/V39A/V105C/A112C/L214E/I285E/C503Q/A547D;
I27E/V39A/V105C/S131N/R134Q/Q205T/L214E/C503Q/A547D/C565N;
I27E/V39A/V105C/R134Q/A153R/P210C/L278D/I285E/C503Q/A547D/A551D;
I27E/V39A/V105C/R134Q/Q205T/P210C/L278D/C503Q/A547D;
I27E/V39A/V105C/R134Q/Q205T/L214E;
I27E/V39A/V105C/R134Q/Q205T/L214E/A551D/C565N;
I27E/V39A/V105C/R134Q/Q205T/L278D/I285E/C503Q/A547D/A551D/C565N;
I27E/V39A/V105C/R134Q/P210C; I27E/V39A/V105C/R134Q/P210C/L214E;
I27E/V39A/V105C/R134Q/P210C/L214E/I285E/A547D;
I27E/V39A/V105C/R134Q/P210C/L214E/C503Q/A551D/C565N;
I27E/V39A/V105C/R134Q/L214E/L278D/A547D/A551D;
I27E/V39A/V105C/R134Q/L214E/I285E/C503Q/A547D/A551D;
I27E/V39A/V105C/R134Q/P266H/C503Q;
I27E/V39A/V105C/R134Q/P266H/C503Q/A547D/A551D;
I27E/V39A/V105C/R134Q/L278D/C503Q/C565N;
I27E/V39A/V105C/R134Q/L278D/I285E/C503Q; I27E/V39A/V105C/R134Q/L278D/A551D;
I27E/V39A/V105C/R134Q/I285E/A547D/A551D; I27E/V39A/V105C/R134Q/C503Q/A551D;
I27E/V39A/V105C/A153R/Q205T/L278D/C503Q/A547D/A551D;
I27E/V39A/V105C/A153R/L214E; I27E/V39A/V105C/A153R/I285E;
I27E/V39A/V105C/A153R/C503Q/A547D/C565N; I27E/V39A/V105C/A153R/A551D/C565N;
I27E/V39A/V105C/Q205T/P210C/L214E/L278D/A547D;
I27E/V39A/V105C/Q205T/P210C/L278D/C503Q;
I27E/V39A/V105C/Q205T/P210C/L278D/A547D;
I27E/V39A/V105C/Q205T/L214E/L278D/C503Q/A547D;
I27E/V39A/V105C/Q205T/L278D/C503Q/A547D;
I27E/V39A/V105C/P210C/I285E/C503Q/A547D/A551D/C565N;
I27E/V39A/V105C/P210C/L214E/P266H/L278D;
I27E/V39A/V105C/L214E/P266H/C503Q/A547D/C565N;
I27E/V39A/V105C/L214E/L278D/L309P/C503Q/A547D/A551D;
I27E/V39A/V105C/L278D/C503Q/A547D/C565N; I27E/V39A/V105C/I285E/A547D;
I27E/V39A/V105C/C503Q/A551D; I27E/V39A/V105C/C503Q/A547D/A551D/C565N;
I27E/V39A/A112C/R134Q/Q205T/P210C/L214E/A551D/C565N;
I27E/V39A/A112C/R134Q/L214E/P266H/A551D;
I27E/V39A/A112C/R134Q/L214E/C503Q/A547D; I27E/V39A/A112C/R134Q/P266H/I285E;
I27E/V39A/A112C/Q205T/L214E/P266H/C503Q/A551D/C565N;

I27E/V39A/A112C/Q205T/L278D/I285E; I27E/V39A/A112C/L214E;
I27E/V39A/A112C/L214E/L278D/C503Q/A547D/A551D; I27E/V39A/A112C/I285E;
I27E/V39A/A112C/A547D; I27E/V39A/R134Q;
I27E/V39A/R134Q/A153R/Q205T/L214E/P266H/C503Q;
I27E/V39A/R134Q/A153R/P210C/L214E/L278D/I285E/A547D/C565N;
I27E/V39A/R134Q/A153R/L214E/P266H/L278D/C503Q/A547D/C565N;
I27E/V39A/R134Q/A153G/L214E/P266H/I285E/C503Q/ A551D/ C565N;
I27E/V39A/R134Q/A153R/L214E/C503Q/A547D; I27E/V39A/R134Q/A153R/L278D;
I27E/V39A/R134Q/A153R/L278D/A547D/A551D; I27E/V39A/R134Q/A153R/A547D;
I27E/V39A/R134Q/Q205T/L214E/P266H/I285E/C503Q/A551D/C565N;
I27E/V39A/R134Q/Q205T/P266H/C503Q/A551D/C565N;
I27E/V39A/R134Q/P210C/L214E/C503Q; I27E/V39A/R134Q/P210C/C503Q/A551D;
I27E/V39A/R134Q/L214E/P266H/A551D;
I27E/V39A/R134Q/L278D/I285E/C503Q/A547D/A551D;
I27E/V39A/R134Q/L278D/C503Q/A547D; I27E/V39A/R134Q/C503Q/A547D;
I27E/V39A/R134Q/A547D/C565N; I27E/V39A/Q205T/L214E/C503Q/C565N;
I27E/V39A/Q205T/P266H/I285E/A547D/A551D/C565N; I27E/V39A/Q205T/P266H/A551D;
I27E/V39A/Q205T/L278D/C503Q/A551D/C565N; I27E/V39A/Q205T/L278D/C503Q/C565N;
I27E/V39A/Q205T/C503Q/A547D/C565N; I27E/V39A/P210C/T212S;
I27E/V39A/P210C/L214E/L278D/C503Q/A551D; I27E/V39A/P210C/L214E/I285E/C503Q/A551D;
I27E/V39A/P210C/P266H/I285E/C503Q/A547D; I27E/V39A/P210C/P266H/C503Q/A551D;
I27E/V39A/L214E; I27E/V39A/L214E/P266H/L278D/C503Q/A547D/A551D/C565N;
I27E/V39A/L214E/L278D/C503Q; I27E/V39A/L214E/L278D/C503Q/A547D/C565N;
I27E/V39A/L214E/C503Q/A551D; I27E/V39A/P266H; I27E/V39A/P266H/L278D;
I27E/V39A/L278D; I27E/V39A/L278D/A547D; I27E/V39A/L278D/I285E/C503Q/A547D;
I27E/V39A/L278D/C503Q/C565N; I27E/V39A/C503Q; I27E/G45D/Q205T/P266H/C565N;
I27E/V105C; I27E/V105C/R134Q/A153R/P210C/L214E/C503Q/A547D;
I27E/V105C/R134Q/A153R/I285E/A547D; I27E/V105C/R134Q/A153R/C503Q;
I27E/V105C/R134Q/Q205T/P210C/C503Q;
I27E/V105C/R134Q/Q205T/L214E/P266H/L278D/C503Q/C565N;
I27E/V105C/Q205T/P266H/C503Q;
I27E/V105C/R134Q/P210C/L214E/P266H/L278D/A551D/C565N;
I27E/V105C/R134Q/P210C/L214E/C503Q/A551D/C565N;
I27E/V105C/R134Q/P210C/P266H/L278D/I285E/C503Q/A551D/C565N;
I27E/V105C/R134Q/L214E/L278D/C503Q/A547D;
I27E/V105C/R134Q/L214E/L278D/C503Q/A547D/A551D/C565N; I27E/V105C/Q205T;
I27E/V105C/Q205T/L214E/P266H; I27E/V105C/Q205T/L214E/P266H/A551D/C565N;

I27E/V105C/Q205T/L214E/L278D/I285E/C503Q/A547D/A551D/C565N;
I27E/V105C/Q205T/C503Q/A547D/A551D/C565N; I27E/V105C/L214E;
I27E/V105C/L214E/P266H/C503Q; I27E/V105C/L214E/I285E/A551D/C565N;
I27E/V105C/L214E/A547D/A551D/C565N; I27E/V105C/L214E/A551D/C565N;
I27E/V105C/P266H; I27E/V105C/P266H/I285E/C503Q/A547D/C565N;
I27E/V105C/L278D/A547D; I27E/V105C/I285E/C503Q/A547D/A551D/C565N;
I27E/V105C/C503Q/A547D/C565N; I27E/V105C/C503Q/A547D/A551D/C565N;
I27E/A112C/R134Q/A153R/L214E/P266H/C503Q;
I27E/A112C/R134Q/L278D/I285E/C503Q/A551D/C565N;
I27E/A112C/R134Q/Q205T/L278D/C503Q; I27E/A112C/R134Q/Q205T/I285E/C503Q;
I27E/A112C/Q205T/P266H/L278D/I285E/C503Q; I27E/A112C/P210C/L214E/C503Q/A547D;
I27E/R134Q; I27E/R134Q/A153R/I285E/C503Q/A547D;
I27E/R134Q/Q205T/I285E/C503Q/A551D; I27E/R134Q/Q205T/P266H/L278D/A547D;
I27E/R134Q/P210C; I27E/R134Q/L214E/C503Q; I27E/R134Q/L214E/C503Q/A547D;
I27E/R134Q/L214E/C503Q/A547D/A551D; I27E/R134Q/L214E/C503Q/C565N;
I27E/R134Q/L278D/I285E/A551D/C565N; I27E/R134Q/I285E/C503Q;
I27E/A153R/L214E/L278D/I285E/A551D/C565N; I27E/A153R/L214E/L278D/A551D;
I27E/Q205T; I27E/Q205T/L214E/L278D/I285E/C503Q/C565N;
I27E/Q205T/L214E/C503Q/A547D/C565N; I27E/Q205T/P266H/L278D/I285E/A551D/C565N;
I27E/Q205T/L278D/A551D; I27E/P210C; I27E/P210C/L214E/C503Q/A547D;
I27E/P210C/L278D/C503Q; I27E/P210C/C503Q; I27E/P210C/C503Q/C565N; I27E/P210C/A551D;
I27E/L214E; I27E/L214E/P266H/L278D/I285E/A551D; I27E/L214E/L278D;
I27E/L214E/L278D/C503Q; I27E/L214E/C503Q; I27E/L214E/C503Q/A547D;
I27E/L214E/C503Q/A547D/C565N; I27E/L214E/A551D; I27E/P266H/L278D/C503Q;
I27E/P266H/A547D/A551D; I27E/L278D/C503Q/A551D; I27E/L278D/C503Q/A551D/C565N;
I27E/A547D/C565N; V39A/G45S/L278D/C503Q/A551D;
V39A/V105C/R134Q/A153R/Q205T/A551D; V39A/V105C/R134Q/P210C/L214E/A551D;
V39A/V105C/R134Q/L214E/C503Q/A547D/A551D; V39A/V105C/A153R/P266H/A547D/A551D;
V39A/V105C/Q205T/C503Q; V39A/V105C/Q205T/A551D; V39A/V105C/P210C/A547D;
V39A/V105C/L214E/P266H/A547D/C565N; V39A/V105C/L214E/I285E/C503Q/A551D/C565N;
V39A/A112C/R134Q/Q205T/L214E/L278D; V39A/A112C/R134Q/L214E/C503Q/A547D/A551D;
V39A/A112C/A153R/Q205T/L278D/C503Q/A547D; V39A/R134Q;
V39A/R134Q/Q205T/L214E/C503Q/C565N; V39A/R134Q/P210C/L214E/A547D/C565N;
V39A/A153R/C503Q/A547D; V39A/Q205T/L278D/A547D/A551D;
V39A/P210C/L214E/L278D/I285E/C503Q/A551D; V39A/P266H;
V39A/P275R/L278D/C503Q/A551D; V39A/C503Q; V39A/C503Q/A551D/C565N; V105C;
V105C/A112C/R134Q/Q205T/L214E/Y492H/C503Q/A547D;

V105C/R134Q/A153R/Q205T/L214E/C503Q; V105C/R134Q/Q205T/L214E/A547D; V105C/R134Q/Q205T/P266H/L278D; V105C/R134Q/L214E/P266H/I285E/C503Q/A551D/C565N; V105C/R134Q/L214E/L278D/C565N; V105C/R134Q/L214E/C503Q/A547D; V105C/R134Q/L214E/C503Q/A547D/A551D; V105C/R134Q/C503Q; V105C/R134Q/C503Q/A547D; V105C/R134Q/C503Q/A547D/C565N; V105C/A153R/Q205T/L214E/P266H/C503Q/A547D; V105C/A153R/Q205T/P266H/I285E/A547D/C565N; V105C/Q205T/P210C/L214E/C503Q/A547D; V105C/Q205T/L214E/L278D; V105C/Q205T/L214E/C503Q/A547D/A551D/C565N; V105C/Q205T/C503Q/A551D; V105C/L214E/P266H/L278D/A547D; V105C/L214E/L278D/C503Q/A547D/A551D; V105C/L214E/I285E; V105C/L214E/I285E/C503Q/A547D/A551D/C565N; V105C/L214E/I285E/A547D/C565N; V105C/L278D/C503Q/A551D; V105C/I285E; V105C/I285E/A547D; V105C/C503Q; V105C/A547D/A551D; A112C/R134Q/A153R/L214E/L278D/I285E/C503Q/A547D/A551D/C565N; A112C/R134Q/L214E/C503Q/A547D/A551D/C565N; A112C/L214E/L278D; A112C/L278D/C503Q/A547D; R134Q/Q205T/L214E/I285E/C503Q/A551D/C565N; R134Q/Q205T/C503Q; R134Q/P210C/L214E/L278D/C503Q/A547D/C565N; R134Q/P210C/L214E/C503Q/A547D/A551D; R134Q/L214E; R134Q/L214E/L278D/C503Q; R134Q/L214E/L278D/C503Q/A551D; R134Q/L214E/I285E/C503Q; R134Q/C503Q; R134Q/C503Q/A547D/A551D; A153R; Q205T/L214E/I285E/C503Q/A551D; Q205T/L214E/I285E/C503Q/C565N; Q205T/L214E/C503Q/A547D/C565N; Q205T/L278D/I285E/A547D/A551D; P210C/L214E; P210C/L214E/P266H; L214E/P266H; L214E/P266H/C503Q/A547D/A551D/C565N; L214E/C503Q/A547D; L214E/A547D; P266H/L278D/C503Q; P266H/C565N; L278D/A547D; C503Q; C503Q/A547D; C503Q/A547D/A551D/C565N; C503Q/A547D/C565N; C503Q/A551D; C503Q/A551D/C565N; A547D; and/or C565N.

[0039] In some additional embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the amino acid residue difference as compared to SEQ ID NO:10, is selected from one or more of the following substitutions or substitution sets: V80I/R134C/P564Q; V121C; A123G; A124G; M125L; L126I/T; L126M/R134L; L127A; A129G/L; N130Q; N130C/M370I R134W; M133R; R134I; R134N/G307C; G135C/S; and/or G135A/A394E.

[0040] In some additional embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the amino acid residue difference as compared to SEQ ID NO:10, is selected from one or more of the following substitutions or substitution sets: G20S/I144L; R43S; L47M/I144L; L47M/R146E; L47M/M147G/A383E; L47M/P157C; Q58H/L143V; Q58K/P157D/G369C; A62S/M147V; S82I/G135C/P157F/W279L; R94C/I149E; T110I/I139R; L118M/L141H; A119E/T156H/A289D; I139M/V; R140D/G/M;

R140N/A199E; R140E/A334S/A551D; L141K/Q/P/T; E142H/P/V; E142D/G371D; L143F/M; I144L/N/V; K145N/Q/R; K145G/P157T; R146H/L; R146W/D191Y; M147A; I149L/R; F150K/L/M; L151M; A153C/G; A153S/H250N; G154R; G154Y/L174M/Q321K/S456I/G483C; T156K/G483C; P157D/F/H/Y; Y158E; V159C/H/L/M; M247I; L319M; and/or Q389K.

[0041] In still some additional embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the amino acid residue difference as compared to SEQ ID NO:10, is selected from one or more of the following substitutions or substitution sets: P117T/Y176Q; V172I/C/L; L174M; S175G; Y176E/I/M/R/V; I177M/V; T178L/A477S; and/or S180C/T.

[0042] In some additional embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the amino acid residue difference as compared to SEQ ID NO:10, is selected from one or more of the following substitutions or substitution sets: R43S/H374K; R43S/H374R; A112S/M370A/A507E; M147I/H374S; S187R/L381V; D191Y/H385N; A232S; Q240K/H374R; A256S/L381N; P275Q/M370S; P275T/H374R; Q332K/Y377M; A334S/H374V; L349M; Q355K/H374S; M370G/I/S; G371H/N/Q/S; M372A/V; H374A/D/G/L/N/R/S/T; H374Q/P396Q; H374R/G417C; L375I; L375M; Y377C/I/N; Y378C/D/E/I/L/N/S; Y378F/P404Q; I379C/H/L/M/N; L381G/V; L381M/Q560K; L382C/H/I/M/S; A383S/V; K384R; H385C/G/N; H385M/P403H; H385S/P403H; D387S; L418M; G425V; A447S; S461G; and/or S525L.

[0043] In some additional embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the amino acid residue difference as compared to SEQ ID NO:10, is selected from one or more of the following substitutions or substitution sets: A24S/F434M; A62S/T433N; S98I; L213M/S438L; Q240K/T433Y; S286R/Y435T; A289S/L431E; S331I; L431C/E/G/P/S/V; L432C/V; T433A/I/L/N/P/Q/R/S/V/W; F434C; Y435L; Y435Q/H446N; G436M; G436D/T; N437E/G/Q; N437T/L538M; S438C/F/M/R/T; I439C/F/L/V; and/or A477S.

[0044] In some additional embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the amino acid residue difference as compared to SEQ ID NO:10, is selected from one or more of the following substitutions or substitution sets: A24E; Q58R/Y475H; A70S/N474E; L104M/V476L; A119E/G365A; L206M; P275Q; G276V; Q292H/A479G; Q355H/I478C; P404T/A477V; I471F/G/K/M/N/R/V/W; F472G; Q473H/K/M/R/S; Q473H/A507S; N474A/H/R/W; N474D/R490H; Y475C/F/L/Q; V476C/I/L; I478N/S; A479G/S; F482C/L; G483C/H/S; G483A/S524I; G483R/G537C; and/or A558S.

[0045] In some additional embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the amino acid residue difference as compared to SEQ ID NO:10, is selected from one or more of the following substitutions or substitution sets: V39A/K115E/M133R/C565N; V39A/M133R/F472G/C503Q/C565N;

V39A/M133R/F472G/C565N; V39A/M133R/C503Q; V39A/M133R/C503Q/C565N; V39A/M147A/Y378E/C503Q/C565N; V39A/M147A/Y378E/C565N; V39A/M147A/L381G/F472G/C503Q/C565N; V39A/M147A/L381G/C503Q/C565N; V39A/M147A/F472G/C503Q/C565N; V39A/M147A/F472G/C565N; V39A/M147A/C565N; V39A/G248C/L381G/F472G/C503Q/C565N; V39A/Y378E/C503Q/C565N; V39A/Y378E/C565N; V39A/L381G; V39A/F472G/C503Q/C565N; V39A/C503Q/C565N; M133R/L381G/C565N; M133R/C503Q; Y378D/C503Q; Y378E/F472G/C503Q/C565N; L381G/F472GC503Q/C565N; and/or F472G/C503Q/C565N.

[0046] In still some further embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the amino acid residue difference as compared to SEQ ID NO:10, is selected from one or more of the following substitutions or substitution sets: I27E/V39A; I27E/V39A/R43L/V105C/A153R/L214E/P266H/L278D/C503Q; I27E/V39A/R43L/L214E/A547D;
I27E/V39A/V105C/A112C/R134Q/L214E/L278D/C503Q/A547D/C565N;
I27E/V39A/V105C/A112C/R134Q/A153R/Q205T/L214E/P266H/L278D/C503Q/A551D;
I27E/V39A/V105C/A112C/Q205T/P210C/P266H/C503Q/A547D;
I27E/V39A/V105C/A112C/Q205T/P266H/I285E/C503Q/A551D;
I27E/V39A/V105C/A112C/L214E/I285E/C503Q/A547D;
I27E/V39A/V105C/S131N/R134Q/Q205T/L214E/C503Q/A547D/C565N;
I27E/V39A/V105C/R134Q/A153R/P210C/L278D/I285E/C503Q/A547D/A551D;
I27E/V39A/V105C/R134Q/Q205T/P210C/L278D/C503Q/A547D;
I27E/V39A/V105C/R134Q/Q205T/L214E;
I27E/V39A/V105C/R134Q/Q205T/L214E/A551D/C565N;
I27E/V39A/V105C/R134Q/Q205T/L278D/I285E/C503Q/A547D/A551D/C565N;
I27E/V39A/V105C/R134Q/P210C; I27E/V39A/V105C/R134Q/P210C/L214E;
I27E/V39A/V105C/R134Q/P210C/L214E/I285E/A547D;
I27E/V39A/V105C/R134Q/P210C/L214E/C503Q/A551D/C565N;
I27E/V39A/V105C/R134Q/L214E/L278D/A547D/A551D;
I27E/V39A/V105C/R134Q/L214E/I285E/C503Q/A547D/A551D;
I27E/V39A/V105C/R134Q/P266H/C503Q;
I27E/V39A/V105C/R134Q/P266H/C503Q/A547D/A551D;
I27E/V39A/V105C/R134Q/L278D/C503Q/C565N;
I27E/V39A/V105C/R134Q/L278D/I285E/C503Q; I27E/V39A/V105C/R134Q/L278D/A551D;
I27E/V39A/V105C/R134Q/I285E/A547D/A551D; I27E/V39A/V105C/R134Q/C503Q/A551D;
I27E/V39A/V105C/A153R/Q205T/L278D/C503Q/A547D/A551D;
I27E/V39A/V105C/A153R/L214E; I27E/V39A/V105C/A153R/I285E;
I27E/V39A/V105C/A153R/C503Q/A547D/C565N; I27E/V39A/V105C/A153R/A551D/C565N;

I27E/V39A/V105C/Q205T/P210C/L214E/L278D/A547D;
I27E/V39A/V105C/Q205T/P210C/L278D/C503Q;
I27E/V39A/V105C/Q205T/P210C/L278D/A547D;
I27E/V39A/V105C/Q205T/L214E/L278D/C503Q/A547D;
I27E/V39A/V105C/Q205T/L278D/C503Q/A547D;
I27E/V39A/V105C/P210C/I285E/C503Q/A547D/A551D/C565N;
I27E/V39A/V105C/P210C/L214E/P266H/L278D;
I27E/V39A/V105C/L214E/P266H/C503Q/A547D/C565N;
I27E/V39A/V105C/L214E/L278D/L309P/C503Q/A547D/A551D;
I27E/V39A/V105C/L278D/C503Q/A547D/C565N; I27E/V39A/V105C/I285E/A547D;
I27E/V39A/V105C/C503Q/A551D; I27E/V39A/V105C/C503Q/A547D/A551D/C565N;
I27E/V39A/A112C/R134Q/Q205T/P210C/L214E/A551D/C565N;
I27E/V39A/A112C/R134Q/L214E/P266H/A551D;
I27E/V39A/A112C/R134Q/L214E/C503Q/A547D; I27E/V39A/A112C/R134Q/P266H/I285E;
I27E/V39A/A112C/Q205T/L214E/P266H/C503Q/A551D/C565N;
I27E/V39A/A112C/Q205T/L278D/I285E; I27E/V39A/A112C/L214E;
I27E/V39A/A112C/L214E/L278D/C503Q/A547D/A551D; I27E/V39A/A112C/I285E;
I27E/V39A/A112C/A547D; I27E/V39A/R134Q;
I27E/V39A/R134Q/A153R/Q205T/L214E/P266H/C503Q;
I27E/V39A/R134Q/A153R/P210C/L214E/L278D/I285E/A547D/C565N;
I27E/V39A/R134Q/A153R/L214E/P266H/L278D/C503Q/A547D/C565N;
I27E/V39A/R134Q/A153G/L214E/P266H/I285E/C503Q/ A551D/ C565N;
I27E/V39A/R134Q/A153R/L214E/C503Q/A547D; I27E/V39A/R134Q/A153R/L278D;
I27E/V39A/R134Q/A153R/L278D/A547D/A551D; I27E/V39A/R134Q/A153R/A547D;
I27E/V39A/R134Q/Q205T/L214E/P266H/I285E/C503Q/A551D/C565N;
I27E/V39A/R134Q/Q205T/P266H/C503Q/A551D/C565N;
I27E/V39A/R134Q/P210C/L214E/C503Q; I27E/V39A/R134Q/P210C/C503Q/A551D;
I27E/V39A/R134Q/L214E/P266H/A551D;
I27E/V39A/R134Q/L278D/I285E/C503Q/A547D/A551D;
I27E/V39A/R134Q/L278D/C503Q/A547D; I27E/V39A/R134Q/C503Q/A547D;
I27E/V39A/R134Q/A547D/C565N; I27E/V39A/Q205T/L214E/C503Q/C565N;
I27E/V39A/Q205T/P266H/I285E/A547D/A551D/C565N; I27E/V39A/Q205T/P266H/A551D;
I27E/V39A/Q205T/L278D/C503Q/A551D/C565N; I27E/V39A/Q205T/L278D/C503Q/C565N;
I27E/V39A/Q205T/C503Q/A547D/C565N; I27E/V39A/P210C/T212S;
I27E/V39A/P210C/L214E/L278D/C503Q/A551D; I27E/V39A/P210C/L214E/I285E/C503Q/A551D;
I27E/V39A/P210C/P266H/I285E/C503Q/A547D; I27E/V39A/P210C/P266H/C503Q/A551D;
I27E/V39A/L214E; I27E/V39A/L214E/P266H/L278D/C503Q/A547D/A551D/C565N;

I27E/V39A/L214E/L278D/C503Q; I27E/V39A/L214E/L278D/C503Q/A547D/C565N;
I27E/V39A/L214E/C503Q/A551D; I27E/V39A/P266H; I27E/V39A/P266H/L278D;
I27E/V39A/L278D; I27E/V39A/L278D/A547D; I27E/V39A/L278D/I285E/C503Q/A547D;
I27E/V39A/L278D/C503Q/C565N; I27E/V39A/C503Q; I27E/G45D/Q205T/P266H/C565N;
I27E/V105C; I27E/V105C/R134Q/A153R/P210C/L214E/C503Q/A547D;
I27E/V105C/R134Q/A153R/I285E/A547D; I27E/V105C/R134Q/A153R/C503Q;
I27E/V105C/R134Q/Q205T/P210C/C503Q;
I27E/V105C/R134Q/Q205T/L214E/P266H/L278D/C503Q/C565N;
I27E/V105C/Q205T/P266H/C503Q;
I27E/V105C/R134Q/P210C/L214E/P266H/L278D/A551D/C565N;
I27E/V105C/R134Q/P210C/L214E/C503Q/A551D/C565N;
I27E/V105C/R134Q/P210C/P266H/L278D/I285E/C503Q/A551D/C565N;
I27E/V105C/R134Q/L214E/L278D/C503Q/A547D;
I27E/V105C/R134Q/L214E/L278D/C503Q/A547D/A551D/C565N; I27E/V105C/Q205T;
I27E/V105C/Q205T/L214E/P266H; I27E/V105C/Q205T/L214E/P266H/A551D/C565N;
I27E/V105C/Q205T/L214E/L278D/I285E/C503Q/A547D/A551D/C565N;
I27E/V105C/Q205T/C503Q/A547D/A551D/C565N; I27E/V105C/L214E;
I27E/V105C/L214E/P266H/C503Q; I27E/V105C/L214E/I285E/A551D/C565N;
I27E/V105C/L214E/A547D/A551D/C565N; I27E/V105C/L214E/A551D/C565N;
I27E/V105C/P266H; I27E/V105C/P266H/I285E/C503Q/A547D/C565N;
I27E/V105C/L278D/A547D; I27E/V105C/I285E/C503Q/A547D/A551D/C565N;
I27E/V105C/C503Q/A547D/C565N; I27E/V105C/C503Q/A547D/A551D/C565N;
I27E/A112C/R134Q/A153R/L214E/P266H/C503Q;
I27E/A112C/R134Q/L278D/I285E/C503Q/A551D/C565N;
I27E/A112C/R134Q/Q205T/L278D/C503Q; I27E/A112C/R134Q/Q205T/I285E/C503Q;
I27E/A112C/Q205T/P266H/L278D/I285E/C503Q; I27E/A112C/P210C/L214E/C503Q/A547D;
I27E/R134Q; I27E/R134Q/A153R/I285E/C503Q/A547D;
I27E/R134Q/Q205T/I285E/C503Q/A551D; I27E/R134Q/Q205T/P266H/L278D/A547D;
I27E/R134Q/P210C; I27E/R134Q/L214E/C503Q; I27E/R134Q/L214E/C503Q/A547D;
I27E/R134Q/L214E/C503Q/A547D/A551D; I27E/R134Q/L214E/C503Q/C565N;
I27E/R134Q/L278D/I285E/A551D/C565N; I27E/R134Q/I285E/C503Q;
I27E/A153R/L214E/L278D/I285E/A551D/C565N; I27E/A153R/L214E/L278D/A551D;
I27E/Q205T; I27E/Q205T/L214E/L278D/I285E/C503Q/C565N;
I27E/Q205T/L214E/C503Q/A547D/C565N; I27E/Q205T/P266H/L278D/I285E/A551D/C565N;
I27E/Q205T/L278D/A551D; I27E/P210C; I27E/P210C/L214E/C503Q/A547D;
I27E/P210C/L278D/C503Q; I27E/P210C/C503Q; I27E/P210C/C503Q/C565N; I27E/P210C/A551D;
I27E/L214E; I27E/L214E/P266H/L278D/I285E/A551D; I27E/L214E/L278D;

I27E/L214E/L278D/C503Q; I27E/L214E/C503Q; I27E/L214E/C503Q/A547D;
I27E/L214E/C503Q/A547D/C565N; I27E/L214E/A551D; I27E/P266H/L278D/C503Q;
I27E/P266H/A547D/A551D; I27E/L278D/C503Q/A551D; I27E/L278D/C503Q/A551D/C565N;
I27E/A547D/C565N; V39A/G45S/L278D/C503Q/A551D;
V39A/V105C/R134Q/A153R/Q205T/A551D; V39A/V105C/R134Q/P210C/L214E/A551D;
V39A/V105C/R134Q/L214E/C503Q/A547D/A551D; V39A/V105C/A153R/P266H/A547D/A551D;
V39A/V105C/Q205T/C503Q; V39A/V105C/Q205T/A551D; V39A/V105C/P210C/A547D;
V39A/V105C/L214E/P266H/A547D/C565N; V39A/V105C/L214E/I285E/C503Q/A551D/C565N;
V39A/A112C/R134Q/Q205T/L214E/L278D; V39A/A112C/R134Q/L214E/C503Q/A547D/A551D;
V39A/A112C/A153R/Q205T/L278D/C503Q/A547D; V39A/R134Q;
V39A/R134Q/Q205T/L214E/C503Q/C565N; V39A/R134Q/P210C/L214E/A547D/C565N;
V39A/A153R/C503Q/A547D; V39A/Q205T/L278D/A547D/A551D;
V39A/P210C/L214E/L278D/I285E/C503Q/A551D; V39A/P266H;
V39A/P275R/L278D/C503Q/A551D; V39A/C503Q; V39A/C503Q/A551D/C565N; V105C;
V105C/A112C/R134Q/Q205T/L214E/Y492H/C503Q/A547D;
V105C/R134Q/A153R/Q205T/L214E/C503Q; V105C/R134Q/Q205T/L214E/A547D;
V105C/R134Q/Q205T/P266H/L278D; V105C/R134Q/L214E/P266H/I285E/C503Q/A551D/C565N;
V105C/R134Q/L214E/L278D/C565N; V105C/R134Q/L214E/C503Q/A547D;
V105C/R134Q/L214E/C503Q/A547D/A551D; V105C/R134Q/C503Q;
V105C/R134Q/C503Q/A547D; V105C/R134Q/C503Q/A547D/C565N;
V105C/A153R/Q205T/L214E/P266H/C503Q/A547D;
V105C/A153R/Q205T/P266H/I285E/A547D/C565N; V105C/Q205T/P210C/L214E/C503Q/A547D;
V105C/Q205T/L214E/L278D; V105C/Q205T/L214E/C503Q/A547D/A551D/C565N;
V105C/Q205T/C503Q/A551D; V105C/L214E/P266H/L278D/A547D;
V105C/L214E/L278D/C503Q/A547D/A551D; V105C/L214E/I285E;
V105C/L214E/I285E/C503Q/A547D/A551D/C565N; V105C/L214E/I285E/A547D/C565N;
V105C/L278D/C503Q/A551D; V105C/I285E; V105C/I285E/A547D; V105C/C503Q;
V105C/A547D/A551D; A112C/R134Q/A153R/L214E/L278D/I285E/C503Q/A547D/A551D/C565N;
A112C/R134Q/L214E/C503Q/A547D/A551D/C565N; A112C/L214E/L278D;
A112C/L278D/C503Q/A547D; R134Q/Q205T/L214E/I285E/C503Q/A551D/C565N;
R134Q/Q205T/C503Q; R134Q/P210C/L214E/L278D/C503Q/A547D/C565N;
R134Q/P210C/L214E/C503Q/A547D/A551D; R134Q/L214E; R134Q/L214E/L278D/C503Q;
R134Q/L214E/L278D/C503Q/A551D; R134Q/L214E/I285E/C503Q; R134Q/C503Q;
R134Q/C503Q/A547D/A551D; A153R; Q205T/L214E/I285E/C503Q/A551D;
Q205T/L214E/I285E/C503Q/C565N; Q205T/L214E/C503Q/A547D/C565N;
Q205T/L278D/I285E/A547D/A551D; P210C/L214E; P210C/L214E/P266H; L214E/P266H;
L214E/P266H/C503Q/A547D/A551D/C565N; L214E/C503Q/A547D; L214E/A547D;

P266H/L278D/C503Q; P266H/C565N; L278D/A547D; C503Q; C503Q/A547D; C503Q/A547D/A551D/C565N; C503Q/A547D/C565N; C503Q/A551D; C503Q/A551D/C565N; A547D; C565N; V80I/R134C/P564Q; V121C; A123G; A124G; M125L; L126I/T; L126M/R134L; L127A; A129G/L; N130Q; N130C/M370I R134W; M133R; R134I; R134N/G307C; G135C/S; G135A/A394E; G20S/I144L; R43S; L47M/I144L; L47M/R146E; L47M/M147G/A383E; L47M/P157C; Q58H/L143V; Q58K/P157D/G369C; A62S/M147V; S82I/G135C/P157F/W279L; R94C/I149E; T110I/I139R; L118M/L141H; A119E/T156H/A289D; I139M/V; R140D/G/M; R140N/A199E; R140E/A334S/A551D; L141K/Q/P/T; E142H/P/V; E142D/G371D; L143F/M; I144L/N/V; K145N/Q/R; K145G/P157T; R146H/L; R146W/D191Y; M147A; I149L/R; F150K/L/M; L151M; A153C/G; A153S/H250N; G154R; G154Y/L174M/Q321K/S456I/G483C; T156K/G483C; P157D/F/H/Y; Y158E; V159C/H/L/M; M247I; L319M; Q389K; P117T/Y176Q; V172I/C/L; L174M; S175G; Y176E/I/M/R/V; I177M/V; T178L/A477S; S180C/T; R43S/H374K; R43S/H374R; A112S/M370A/A507E; M147I/H374S; S187R/L381V; D191Y/H385N; A232S; Q240K/H374R; A256S/L381N; P275Q/M370S; P275T/H374R; Q332K/Y377M; A334S/H374V; L349M; Q355K/H374S; M370G/I/S; G371H/N/Q/S; M372A/V; H374A/D/G/L/N/R/S/T; H374Q/P396Q; H374R/G417C; L375I; L375M; Y377C/I/N; Y378C/D/E/I/L/N/S; Y378F/P404Q; I379C/H/L/M/N; L381G/V; L381M/Q560K; L382C/H/I/M/S; A383S/V; K384R; H385C/G/N; H385M/P403H; H385S/P403H; D387S; L418M; G425V; A447S; S461G; S525L; A24S/F434M; A62S/T433N; S98I; L213M/S438L; Q240K/T433Y; S286R/Y435T; A289S/L431E; S331I; L431C/E/G/P/S/V; L432C/V; T433A/I/L/N/P/Q/R/S/V/W; F434C; Y435L; Y435Q/H446N; G436M; G436D/T; N437E/G/Q; N437T/L538M; S438C/F/M/R/T; I439C/F/L/V; A477S; A24E; Q58R/Y475H; A70S/N474E; L104M/V476L; A119E/G365A; L206M; P275Q; G276V; Q292H/A479G; Q355H/I478C; P404T/A477V; I471F/G/K/M/N/R/V/W; F472G; Q473H/K/M/R/S; Q473H/A507S; N474A/H/R/W; N474D/R490H; Y475C/F/L/Q; V476C/I/L; I478N/S; A479G/S; F482C/L; G483C/H/S; G483A/S524I; G483R/G537C; A558S; V39A/K115E/M133R/C565N; V39A/M133R/F472G/C503Q/C565N; V39A/M133R/F472G/C565N; V39A/M133R/C503Q; V39A/M133R/C503Q/C565N; V39A/M147A/Y378E/C503Q/C565N; V39A/M147A/Y378E/C565N; V39A/M147A/L381G/F472G/C503Q/C565N; V39A/M147A/L381G/C503Q/C565N; V39A/M147A/F472G/C503Q/C565N; V39A/M147A/F472G/C565N; V39A/M147A/C565N; V39A/G248C/L381G/F472G/C503Q/C565N; V39A/Y378E/C503Q/C565N; V39A/Y378E/C565N; V39A/L381G; V39A/F472G/C503Q/C565N; V39A/C503Q/C565N; M133R/L381G/C565N; M133R/C503Q; Y378D/C503Q; Y378E/F472G/C503Q/C565N; L381G/F472GC503Q/C565N; and/or F472G/C503Q/C565N.

[0047] The present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about

96%, at least about 97%, at least about 98%, at least about 99%, or greater sequence identity to reference sequence SEQ ID NO:26 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:26 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:26.

[0048] In some embodiments, the present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater sequence identity to reference sequence SEQ ID NO:26 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:26 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:26.

[0049] In some additional embodiments, the present invention provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity comprising: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:26 or a functional fragment thereof; b) a polypeptide sequence comprising at least one amino acid residue difference as compared to SEQ ID NO:26 or the functional fragment thereof at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced sensitivity to proteolysis, iii) increased tolerance to acidic pH, iv) reduced aggregation, v) reduced immunogenicity, or a combination of any of i), ii), iii), iv), or v), as compared to the reference sequence SEQ ID NO:26. In some embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity of the present invention comprise an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:26, and at least one amino acid residue difference as compared to SEQ ID NO:126, and that exhibit at least one improved property selected from enhanced catalytic activity, reduced sensitivity to proteolysis, increased tolerance to acidic pH, reduced aggregation, and/or reduced immunogenicity, as compared to SEQ ID NO:26. In some embodiments of the engineered polypeptides, the amino acid residue difference as compared to SEQ ID NO:26 is selected from one or more of the following substitutions or substitution sets A24E/G381L; L127V; A129I/V; S131C/T; H132L/S; R134C/F/H/K; R134H/Y378E/G381L; R134H/Y378E/G381L/V388T; R134H/V388T; A136K; A289S; M372L; H374G/M/Q; G381A/C/F/I/L/M/N/Q/S/T; A383C/M; V388C/T; L431M; and/or L563M.

[0050] In some embodiments, the engineered polypeptide having phenylalanine ammonia lyase (PAL) activity of the present invention has at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to reference sequence SEQ ID NO:4. In some embodiments, the engineered polypeptide having phenylalanine ammonia lyase (PAL) activity of the present invention has at least about 90% sequence identity to reference sequence SEQ ID NO:4, while in some further embodiments, the engineered polypeptide has at least about 95% sequence identity to reference sequence SEQ ID NO:4. In some embodiments, the engineered polypeptide having phenylalanine ammonia lyase (PAL) activity of the present invention has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to reference sequence SEQ ID NO:4. In some embodiments, the engineered polypeptide having phenylalanine ammonia lyase (PAL) activity of the present invention has at least 90% sequence identity to reference sequence SEQ ID NO:4, while in some further embodiments, the engineered polypeptide has at least 95% sequence identity to reference sequence SEQ ID NO:4. In some further embodiments, the engineered polypeptides comprise functional fragments of polypeptides (e.g., any of the variant provided in the Tables, herein) having phenylalanine ammonia lyase (PAL) activity of the present invention.

[0051] In some embodiments, the engineered polypeptide having phenylalanine ammonia lyase (PAL) activity of the present invention has at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26. In some embodiments, the engineered polypeptide having phenylalanine ammonia lyase (PAL) activity of the present invention has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26. In some embodiments, the engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity comprise an amino acid sequence having at least about 90% sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26. In some embodiments, the engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity comprises an amino acid sequence having at least about 99% sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26, or a functional fragment thereof. In some further embodiments, the engineered polypeptides comprise functional fragments of polypeptides (e.g., functional fragments of SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26, as well as any of the variants provided in the Tables, herein) having phenylalanine ammonia lyase (PAL) activity of the present invention.

[0052] The present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, wherein the engineered polypeptides are variant PALs provided in any of Tables 2-1 through 2-5 and/or Tables 9-1 through 9-7.

[0053] In some embodiments, the engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity is an *Anabaena variabilis* enzyme. In some additional embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity are thermostable. In some embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity are resistant to proteolysis. In some additional embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity are resistant to proteolysis by at least one digestive tract enzyme. In some further embodiments, the engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity are resistant to proteolysis by chymotrypsin, trypsin, carboxypeptidases, and/or elastases. In some further embodiments, the engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity is acid stable.

[0054] The present invention also provides engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity that are deimmunized. In some embodiments, the deimmunized engineered polypeptides comprise an amino acid sequence having at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or greater, sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26. In some additional embodiments, the deimmunized engineered polypeptides comprise an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater, sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26. In some embodiments, the deimmunized engineered polypeptides comprise an amino acid sequence having at least 95% sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26. In some embodiments, the deimmunized engineered polypeptides comprise an amino acid sequence having 95% sequence identity to SEQ ID NO:6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and/or 26.

[0055] In still some further embodiments, the present invention provides purified engineered polypeptides having phenylalanine ammonia lyase (PAL) activity.

[0056] The present invention also provides polynucleotide sequences encoding at least one engineered polypeptide having phenylalanine ammonia lyase (PAL), as set forth herein. In some embodiments, the polynucleotide sequence is operably linked to a control sequence. In some additional embodiments, the polynucleotide sequence is codon-optimized.

[0057] The present invention also provides expression vectors comprising at least one polynucleotide sequence encoding at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity, as provided herein. In some embodiments, the expression vector further comprises at least one control sequence. In some embodiments, the control sequence is a promoter. In some additional embodiments, the promoter is a heterologous promoter.

[0058] The present invention also provides host cells transformed with at least one polynucleotide sequence encoding at least one engineered polypeptides having phenylalanine ammonia-lyase (PAL) activity, and/or at least one expression vector comprising at least one polynucleotide sequence

encoding at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity and at least one control sequence. In some embodiments, the host cells comprise at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity that is codon-optimized. In some embodiments, the host cell is *E. coli*.

[0059] The present invention also provides methods of producing at least one engineered PAL polypeptide in a host cell comprising culturing a host cell comprising at least one polynucleotide encoding at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity, and/or at least one expression vector comprising at least one polynucleotide sequence encoding at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity and at least one control sequence, under suitable culture conditions, such that the engineered PAL polypeptide is produced. In some embodiments, the methods further comprise the step of recovering at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) from the culture and/or host cells. In some further embodiments, the methods further comprise the step of purifying at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL).

[0060] The present invention also provides compositions comprising at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity as provided herein. In some embodiments, the composition is a pharmaceutical composition. In some embodiments, the composition is a dietary and/or nutritional supplement. In some further embodiments, the pharmaceutical compositions further comprise at least one pharmaceutically acceptable excipient and/or carrier. In some additional embodiments, the composition is suitable for the treatment of phenylketonuria. In some further embodiments, the pharmaceutical composition is suitable for oral administration to a human. In some embodiments, the composition is in the form of a pill, tablet, capsule, gelcap, liquid, or emulsion. In yet some further embodiments, the pill, tablet, capsule, or gelcap further comprises an enteric coating. In some additional embodiments, the pharmaceutical composition is suitable for parenteral injection into a human. In some embodiments, the pharmaceutical composition is coadministered with at least one additional therapeutically effective compound. In some further embodiments, the pharmaceutical composition comprises at least one additional therapeutically effective compound. In some additional embodiments, the pharmaceutical composition is present in a dietary and/or nutritional supplement.

[0061] The present invention also provides methods for treating and/or preventing the symptoms of phenylketonuria in a subject, comprising providing a subject having phenylketonuria, and providing at least one composition provided herein to the subject. In some embodiments, the composition comprises a pharmaceutical composition, while in some alternative embodiments, the composition comprises a dietary/nutritional supplement. In some embodiments of the methods, the symptoms of phenylketonuria are ameliorated. In some additional embodiments, the treated subject is able to eat a diet that is less restricted in its methionine, phenylalanine, and/or tyrosine content than diets required by subjects exhibiting the symptoms of phenylalanine. In some embodiments, the treated subject (i.e.,

a subject who has been provided with at least one composition comprising at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity as provided herein) is able to eat a diet that is less restricted in its methionine, phenylalanine and/or tyrosine content than diets required by subjects who have not been provided at least one composition as provided herein. In some embodiments, the composition provided to the subjects comprises a pharmaceutical composition, while in some alternative embodiments, the composition comprises a dietary/nutritional supplement. The present invention also provides treated subjects, wherein the subject has been administered at least one composition and/or pharmaceutical composition comprising at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity as provided herein. In some embodiments, the subject is an animal selected from primates, rodents, and lagamorphs. In some additional embodiments, the subject is a mouse. In some further embodiments, the subject is a human. In still some further embodiments, the subject is a human infant or child, while in some alternative embodiments, the subject is a human adult or young adult. I

[0062] The present invention also provides uses of the compositions comprising at least one engineered polypeptide having phenylalanine ammonia-lyase (PAL) activity provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0063] Figure 1 provides an alignment of wild-type PAL protein sequences: *Anabaena variabilis* PAL (NCBI YP_ 324488.1 (SEQ ID NO:4)); *Nostoc punctiforme* phenylalanine/histidine ammonia lyase "NpPHAL" (NCBI YP_001865631.1 (SEQ ID NO:30); *Rivularia sp.* histidine ammonia-lyase "RspHAL" (NCBI YP_007056096.1 (SEQ ID NO:31); *Oscillatoria sp.* histidine ammonia-lyase "Osp HAL" (NCBI YP_07108482.1(SEQ ID NO:32); and *Gloeocapsa sp.* histidine ammonia-lyase "GspHAL" (NCBI YP_007127054.1) (SEQ ID NO:33).

[0064] Figure 2A shows the reduced sensitivity to proteolysis (expressed as chymotrypsin and trypsin tolerance) as compared to wild-type AvPAL tested at pH 7.0 for Variant No. 22 (SEQ ID NO:8), Variant No. 30 (SEQ ID NO:6) and Variant No. 36 (SEQ ID NO:10) as further described in Example 4.

[0065] Figure 2B provides a graph showing an increased tolerance to acidic pH as compared to wild-type AvPAL tested at pH 4.0 to 5.2, for Variant Nos. 22, 30 and 36 as further described in Example 4.

[0066] Figure 3 provides K_M results for wild-type PAL and Variant No. 36.

[0067] Figure 4 provides data showing the amino acid specificity of wild-type PAL and Variant No. 36.

[0068] Figure 5 provides results showing the relative stability of wild-type PAL and Variant No. 36 exposed to human chymotrypsin and trypsin.

[0069] Figure 6 provides results showing the relative stability of wild-type PAL, and Variant Nos. 36, 42, and 43 exposed to porcine pancreatic extract.

[0069a] Figure 7 provides a graph showing the results obtained for Example 5.

[0069b] Figure 8 provides a graph showing the results obtained for Example 8.

DESCRIPTION OF THE INVENTION

[0070] The present invention provides engineered PAL polypeptides, mutants, biologically active fragments and analogues thereof, and pharmaceutical and industrial compositions comprising the same.

[0071] The invention provides engineered phenylalanine ammonia-lyase (PAL) polypeptides and compositions thereof, as well as polynucleotides encoding the engineered phenylalanine ammonia-lyase (PAL) polypeptides. In some embodiments, the engineered PAL polypeptides are optimized to provide enhanced catalytic activity, as well as reduced sensitivity to proteolysis and increased tolerance to acidic pH levels. In some embodiments the engineered PAL polypeptides are deimmunized. The invention also relates to the use of the compositions comprising the engineered PAL polypeptides for therapeutic and industrial purposes.

Abbreviations and Definitions:

[0072] Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Generally, the nomenclature used herein and the laboratory procedures of cell culture, molecular genetics, microbiology, organic chemistry, analytical chemistry and nucleic acid chemistry described below are those well-known and commonly employed in the art. Such techniques are well- known and described in numerous texts and reference works well known to those of skill in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[0073] Although any suitable methods and materials similar or equivalent to those described herein find use in the practice of the present invention, some methods and materials are described herein. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art. Accordingly, the terms defined immediately below are more fully described by reference to the application as a whole. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[0074] Also, as used herein, the singular "a", "an," and "the" include the plural references, unless the context clearly indicates otherwise.

[0075] Numeric ranges are inclusive of the numbers defining the range. Thus, every numerical range disclosed herein is intended to encompass every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein. It is

also intended that every maximum (or minimum) numerical limitation disclosed herein includes every

lower (or higher) numerical limitation, as if such lower (or higher) numerical limitations were expressly written herein.

[0076] The term "about" means an acceptable error for a particular value. In some instances "about" means within 0.05%, 0.5%, 1.0%, or 2.0%, of a given value range. In some instances, "about" means within 1, 2, 3, or 4 standard deviations of a given value.

[0077] Furthermore, the headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the application as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the application as a whole. Nonetheless, in order to facilitate understanding of the invention, a number of terms are defined below.

[0078] Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

[0079] As used herein, the term "comprising" and its cognates are used in their inclusive sense (*i.e.*, equivalent to the term "including" and its corresponding cognates).

[0080] "EC" number refers to the Enzyme Nomenclature of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). The IUBMB biochemical classification is a numerical classification system for enzymes based on the chemical reactions they catalyze.

[0081] "ATCC" refers to the American Type Culture Collection whose biorepository collection includes genes and strains.

[0082] "NCBI" refers to National Center for Biological Information and the sequence databases provided therein.

[0083] As used herein, the term "phenylalanine ammonia-lyase (PAL) polypeptide" refers to a class of enzymes within the aromatic amino acid lyase family (EC 4.3.1.23, EC 4.3.1.24 and EC4.3.1.25) which also includes histidine ammonia-lyase, and tyrosine ammonia-lyase. The PAL polypeptides are also sometimes referred to as phenylalanine/tyrosine ammonia-lyases because some PAL enzymes may use tyrosine as well as phenylalanine as a substrate. However, the AvPAL and variants disclosed and claimed herein do not use tyrosine as a substrate. PAL polypeptides catalyze the conversion of L-phenylalanine to trans-cinnamic acid and ammonia. PAL activity refers to the enzymatic activity of PAL polypeptides. In some preferred embodiments, a PAL enzyme also contains the cofactor 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO). This cofactor maybe required for catalytic activity and is formed by cyclization and dehydration of a conserved active site Ala167-Ser168-Gly169 tripeptide segment.

[0084] "Protein," "polypeptide," and "peptide" are used interchangeably herein to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

[0085] "Amino acids" are referred to herein by either their commonly known three-letter symbols or by the one-letter symbols recommended by IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single letter codes.

[0086] The term "engineered," "recombinant," "non-naturally occurring," and "variant," when used with reference to a cell, a polynucleotide or a polypeptide refers to a material or a material corresponding to the natural or native form of the material that has been modified in a manner that would not otherwise exist in nature or is identical thereto but produced or derived from synthetic materials and/or by manipulation using recombinant techniques.

[0087] As used herein, "wild-type" and "naturally-occurring" refer to the form found in nature. For example a wild-type polypeptide or polynucleotide sequence is a sequence present in an organism that can be isolated from a source in nature and which has not been intentionally modified by human manipulation.

[0088] "Deimmunized" as used herein, refers to the manipulation of a protein to create a variant that is not as immunogenic as the wild-type or reference protein. In some embodiments, the deimmunization is complete, in that the variant protein does not stimulate an immune response in patients to whom the variant protein is administered. This response can be measured by various methods including but not limited to, the presence or abundance of neutralizing (i.e., anti-drug antibodies), the presence of an anaphylactic response, or the prevalence or intensity of cytokine release upon administration of the protein. In some embodiments, the variant protein is less immunogenic than the wild-type or reference protein. In some embodiments, deimmunization involves modifications to proteins (e.g., epitopes) that are recognized by T-cell receptors. In some embodiments, the T-cell epitopes are removed from a wild-type or reference protein in order to produce a deimmunized variant protein. In some embodiments, the deimmunized protein shows lower levels of response in biochemical and cell-biological predictors of human immunological responses including dendritic-cell T-cell activation assays, or human leukocyte antigen (HLA) peptide binding assays.

[0089] "Coding sequence" refers to that part of a nucleic acid (e.g., a gene) that encodes an amino acid sequence of a protein.

[0090] The term "percent (%) sequence identity" is used herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence for optimal alignment of the two sequences. The percentage may be calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Alternatively, the percentage may be calculated by

determining the number of positions at which either the identical nucleic acid base or amino acid residue occurs in both sequences or a nucleic acid base or amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Those of skill in the art appreciate that there are many established algorithms available to align two sequences. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman (Smith and Waterman, Adv. Appl. Math., 2:482 [1981]), by the homology alignment algorithm of Needleman and Wunsch (Needleman and Wunsch, J. Mol. Biol., 48:443 [1970]), by the search for similarity method of Pearson and Lipman (Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 [1988]), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package), or by visual inspection, as known in the art. Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity include, but are not limited to the BLAST and BLAST 2.0 algorithms (See e.g., Altschul et al., J. Mol. Biol., 215: 403-410 [1990]; and Altschul *et al.*, Nucleic Acids Res., 3389-3402 [1977]). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length "W" in the query sequence, which either match or satisfy some positive-valued threshold score "T," when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (See, Altschul et al, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters "M" (reward score for a pair of matching residues; always >0) and "N" (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity "X" from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (See e.g., Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 [1989]). Exemplary determination of sequence alignment and % sequence identity can employ the BESTFIT or GAP programs in the GCG Wisconsin Software package (Accelrys, Madison WI), using default parameters provided.

[0091] "Reference sequence" refers to a defined sequence used as a basis for a sequence comparison. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence is at least 20 nucleotide or amino acid residues in length, at least 25 residues in length, at least 50 residues in length, at least 100 residues in length or the full length of the nucleic acid or polypeptide. Since two polynucleotides or polypeptides may each (1) comprise a sequence (*i.e.*, a portion of the complete sequence) that is similar between the two sequences, and (2) may further comprise a sequence that is divergent between the two sequences, sequence comparisons between two (or more) polynucleotides or polypeptide are typically performed by comparing sequences of the two polynucleotides or polypeptides over a "comparison window" to identify and compare local regions of sequence similarity. In some embodiments, a "reference sequence" can be based on a primary amino acid sequence, where the reference sequence is a sequence that can have one or more changes in the primary sequence. For instance, the phrase "reference sequence based on SEQ ID NO:4 having a valine at the residue corresponding to X39" refers to a reference sequence in which the corresponding residue at position X39 in SEQ ID NO:4 (e.g., an alanine), has been changed to valine.

[0092] "Comparison window" refers to a conceptual segment of at least about 20 contiguous nucleotide positions or amino acids residues wherein a sequence may be compared to a reference sequence of at least 20 contiguous nucleotides or amino acids and wherein the portion of the sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The comparison window can be longer than 20 contiguous residues, and includes, optionally 30, 40, 50, 100, or longer windows.

[0093] "Corresponding to", "reference to," and "relative to" when used in the context of the numbering of a given amino acid or polynucleotide sequence refer to the numbering of the residues of a specified reference sequence when the given amino acid or polynucleotide sequence is compared to the reference sequence. In other words, the residue number or residue position of a given polymer is designated with respect to the reference sequence rather than by the actual numerical position of the residue within the given amino acid or polynucleotide sequence. For example, a given amino acid sequence, such as that of an engineered PAL, can be aligned to a reference sequence by introducing gaps to optimize residue matches between the two sequences. In these cases, although the gaps are present, the numbering of the residue in the given amino acid or polynucleotide sequence is made with respect to the reference sequence to which it has been aligned.

[0094] "Amino acid difference" and "residue difference" refer to a difference in the amino acid residue at a position of a polypeptide sequence relative to the amino acid residue at a corresponding position in a reference sequence. The positions of amino acid differences generally are referred to herein as "Xn," where n refers to the corresponding position in the reference sequence upon which the residue difference is based. For example, a "residue difference at position X91 as compared to SEQ

ID NO:4" refers to a difference of the amino acid residue at the polypeptide position corresponding to position 91 of SEQ ID NO:4. Thus, if the reference polypeptide of SEQ ID NO:4 has a alanine at position 91, then a "residue difference at position X91 as compared to SEQ ID NO:4" refers to an amino acid substitution of any residue other than alanine at the position of the polypeptide corresponding to position 91 of SEQ ID NO:4. In most instances herein, the specific amino acid residue difference at a position is indicated as "XnY" where "Xn" specified the corresponding residue and position of the reference polypeptide (as described above), and "Y" is the single letter identifier of the amino acid found in the engineered polypeptide (*i.e.*, the different residue than in the reference polypeptide). In some instances (*e.g.*, in the Tables in the Examples), the present disclosure also provides specific amino acid differences denoted by the conventional notation "AnB", where A is the single letter identifier of the residue in the reference sequence, "n" is the number of the residue position in the reference sequence, and B is the single letter identifier of the residue substitution in the sequence of the engineered polypeptide. In some instances, a polypeptide of the present disclosure can include one or more amino acid residue differences relative to a reference sequence, which is indicated by a list of the specified positions where residue differences are present relative to the reference sequence. In some embodiments, where more than one amino acid can be used in a specific residue position of a polypeptide, the various amino acid residues that can be used are separated by a "/" (*e.g.*, X307G/X307Q or X307G/Q). The present disclosure includes engineered polypeptide sequences comprising one or more amino acid differences that include either/or both conservative and non-conservative amino acid substitutions.

[0095] The terms "amino acid substitution set" and "substitution set" refers to a group of amino acid substitutions within a polypeptide sequence. In some embodiments, substitution sets comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions. In some embodiments, a substitution set refers to the set of amino acid substitutions that is present in any of the variant AvPAL polypeptides listed in any of the Tables in the Examples. For example, the substitution set present in Variant 36 is A39V/A91V/N290G/H307G/L407V/ T524S, wherein the amino acid positions are relative to SEQ ID NO:4.

[0096] "Conservative amino acid substitution" refers to a substitution of a residue with a different residue having a similar side chain, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar defined class of amino acids. By way of example and not limitation, an amino acid with an aliphatic side chain may be substituted with another aliphatic amino acid (*e.g.*, alanine, valine, leucine, and isoleucine); an amino acid with hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain (*e.g.*, serine and threonine); an amino acids having aromatic side chains is substituted with another amino acid having an aromatic side chain (*e.g.*, phenylalanine, tyrosine, tryptophan, and histidine); an amino acid with a basic side chain is substituted with another amino acid with a basis side chain (*e.g.*, lysine and arginine); an amino acid with an acidic side chain is substituted with another amino acid with an

acidic side chain (*e.g.*, aspartic acid or glutamic acid); and a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively.

[0097] "Non-conservative substitution" refers to substitution of an amino acid in the polypeptide with an amino acid with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and affect: (a) the structure of the peptide backbone in the area of the substitution (*e.g.*, proline for glycine); (b) the charge or hydrophobicity; and/or (c) the bulk of the side chain. By way of example and not limitation, exemplary non-conservative substitutions include an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

[0098] "Deletion" refers to modification to the polypeptide by removal of one or more amino acids from the reference polypeptide. Deletions can comprise removal of 1 or more amino acids, 2 or more amino acids, 5 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, or up to 20% of the total number of amino acids making up the reference enzyme while retaining enzymatic activity and/or retaining the improved properties of an engineered transaminase enzyme. Deletions can be directed to the internal portions and/or terminal portions of the polypeptide. In various embodiments, the deletion can comprise a continuous segment or can be discontinuous.

[0099] "Insertion" refers to modification to the polypeptide by addition of one or more amino acids from the reference polypeptide. Insertions can be in the internal portions of the polypeptide, or to the carboxy or amino terminus. Insertions as used herein include fusion proteins as is known in the art. The insertion can be a contiguous segment of amino acids or separated by one or more of the amino acids in the naturally occurring polypeptide.

[0100] The terms "functional fragment" and "biologically active fragment" are used interchangeably herein, to refer to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion(s) and/or internal deletions, but where the remaining amino acid sequence is identical to the corresponding positions in the sequence to which it is being compared (e.g., a full length engineered PAL of the present invention) and that retains substantially all of the activity of the full-length polypeptide.

[0101] "Isolated polypeptide" refers to a polypeptide which is substantially separated from other contaminants that naturally accompany it (*e.g.*, protein, lipids, and polynucleotides). The term embraces polypeptides which have been removed or purified from their naturally-occurring environment or expression system (*e.g.*, host cell or *in vitro* synthesis). The recombinant PAL polypeptides may be present within a cell, present in the cellular medium, or prepared in various forms, such as lysates or isolated preparations. As such, in some embodiments, the recombinant PAL polypeptides provided herein are isolated polypeptides.

[0102] "Substantially pure polypeptide" refers to a composition in which the polypeptide species is the predominant species present (*i.e.*, on a molar or weight basis it is more abundant than any other

individual macromolecular species in the composition), and is generally a substantially purified composition when the object species comprises at least about 50 percent of the macromolecular species present by mole or % weight. Generally, a substantially pure PAL composition will comprise about 60% or more, about 70% or more, about 80% or more, about 90% or more, about 95% or more, and about 98% or more of all macromolecular species by mole or % weight present in the composition. In some embodiments, the object species is purified to essential homogeneity (*i.e.*, contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species. In some embodiments, the isolated recombinant PAL polypeptides are substantially pure polypeptide compositions.

[0103] "Improved enzyme property" refers to an engineered PAL polypeptide that exhibits an improvement in any enzyme property as compared to a reference PAL polypeptide, such as a wild-type PAL polypeptide (e.g., AvPAL wild-type having SEQ ID NO:4) or another engineered PAL polypeptide. Improved properties include but are not limited to such properties as increased protein expression, increased thermoactivity, increased thermostability, increased pH activity, increased stability, increased enzymatic activity, increased substrate specificity and/or affinity, increased specific activity, increased resistance to substrate and/or end-product inhibition, increased chemical stability, improved chemoselectivity, improved solvent stability, increased tolerance to acidic pH, increased tolerance to proteolytic activity (i.e., reduced sensitivity to proteolysis), reduced aggregation, increased solubility, reduced immunogenicity, and altered temperature profile.

[0104] "Increased enzymatic activity" and "enhanced catalytic activity" refer to an improved property of the engineered PAL polypeptides, which can be represented by an increase in specific activity (*e.g.*, product produced/time/weight protein) and/or an increase in percent conversion of the substrate to the product (*e.g.*, percent conversion of starting amount of substrate to product in a specified time period using a specified amount of PAL) as compared to the reference PAL enzyme (e.g., wild-type AvPAL and/or another engineered AvPAL). Exemplary methods to determine enzyme activity are provided in the Examples. Any property relating to enzyme activity may be affected, including the classical enzyme properties of K_m, V_{max} or k_{cat}, changes of which can lead to increased enzymatic activity. Improvements in enzyme activity can be from about 1.1 fold the enzymatic activity of the corresponding wild-type enzyme, to as much as 2-fold, 5-fold, 10-fold, 20-fold, 25-fold, 50-fold, 75-fold, 100-fold, 150-fold, 200-fold or more enzymatic activity than the naturally occurring PAL or another engineered PAL from which the PAL polypeptides were derived.

[0105] In some embodiments, the engineered PAL polypeptides have a k_{cat} of at least 0.1/sec, at least 0.2/sec, at least 0.3/sec, at least 0.5/sec, at least 1.0/sec and in some preferred embodiments greater than 1.0/sec. In some embodiments, the K_m is in the range of about 1μm to about 5mM; in the range of about 5μm to about 2mM; in the range of about10μm to about 2mM; or in the range of about 10μm to

about 1mM. In some specific embodiments, the engineered PAL enzyme exhibits improved enzymatic activity in the range of 1.5 to 10 fold, 1.5 to 25 fold, 1.5 to 50 fold, 1.5 to 100 fold or greater, than that of the reference PAL enzyme. PAL activity can be measured by any standard assay known in the art, (e.g., by monitoring changes in spectrophotometric properties of reactants or products). In some embodiments, the amount of products produced is measured by High-Performance Liquid Chromatography (HPLC) separation combined with UV absorbance or fluorescent detection directly or following o-phthaldialdehyde (OPA) derivatization. In some embodiments, comparisons of enzyme activities are made using a defined preparation of enzyme, a defined assay under a set condition, and one or more defined substrates, as further described in detail herein. Generally, when lysates are compared, the numbers of cells and the amount of protein assayed are determined as well as use of identical expression systems and identical host cells, in order to minimize variations in amount of enzyme produced by the host cells and present in the lysates.

[0106] The term "improved tolerance to acidic pH" means that a recombinant PAL according to the invention exhibits increased stability (i.e., higher retained activity at about pH 7.0, after exposure to acidic pH for a specified period of time [1 hour, up to 24 hours]) as compared to a reference PAL.

[0107] "Physiological pH" as used herein means the pH range generally found in a subject's (e.g., human) small intestine. There normally is a gradient pH from the pyloric valve to the large intestine, in the range of about 6.0 to 7.5.

[0108] The term "acidic pH" used with reference to improved stability to acidic pH conditions or increased tolerance to acidic pH means a pH range of about 1.5 to 6.8.

[0109] The terms "proteolytic activity" and "proteolysis" used interchangeably herein refer to the breakdown of proteins into smaller polypeptides or amino acids. The breakdown of proteins is generally the result of hydrolysis of the peptide bond by protease (proteinase) enzymes. Protease enzymes include but are not limited to pepsin, trypsin, chymotrypsin, elastase; carboxypeptidase A and B, and peptidases (e.g., amino peptidase, dipeptidase and enteropeptidase).

[0110] The phrases "reducing sensitivity to proteolysis" and "reducing proteolytic sensitivity" are used interchangeably herein mean that an engineered PAL polypeptide according to the invention will have a higher enzyme activity compared to a reference PAL in a standard assay (e.g., as disclosed in the Examples) after treatment with one or more proteases.

[0111] "Aggregation" means clumping or precipitation of a PAL polypeptide. Aggregation can lead to inactivation of the enzyme. The term "reduced aggregation" means an engineered PAL polypeptide will be less prone to aggregation, as compared to a reference PAL. Methods for assessing aggregation are known in the art, including but not limited to the use of fluorescent microscopy with appropriate dyes (e.g., thioflavin T or Nile Red), dynamic light scattering, flow cytometry with appropriate dyes (e.g., Bodipy), filtration and analysis by SDS-PAGE, and/or Western blotting, fluorescent correlation spectroscopy, and electron microscopy. There are commercially available kits to assess aggregation (e.g., the ProteoStat® Protein Aggregation Assay kit [Enzo]).

[0112] "Conversion" refers to the enzymatic conversion (or biotransformation) of substrate(s) to the corresponding product(s). "Percent conversion" refers to the percent of the substrate that is converted to the product within a period of time under specified conditions. Thus, the "enzymatic activity" or "activity" of a PAL polypeptide can be expressed as "percent conversion" of the substrate to the product in a specific period of time.

[0113] "Hybridization stringency" relates to hybridization conditions, such as washing conditions, in the hybridization of nucleic acids. Generally, hybridization reactions are performed under conditions of lower stringency, followed by washes of varying but higher stringency. The term "moderately stringent hybridization" refers to conditions that permit target-DNA to bind a complementary nucleic acid that has about 60% identity, preferably about 75% identity, about 85% identity to the target DNA, with greater than about 90% identity to target-polynucleotide. Exemplary moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42°C, followed by washing in 0.2×SSPE, 0.2% SDS, at 42°C. "High stringency hybridization" refers generally to conditions that are about 10°C or less from the thermal melting temperature T_m as determined under the solution condition for a defined polynucleotide sequence. In some embodiments, a high stringency condition refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C (*i.e.*, if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in conditions equivalent to 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42°C, followed by washing in 0.1×SSPE, and 0.1% SDS at 65°C. Another high stringency condition is hybridizing in conditions equivalent to hybridizing in 5X SSC containing 0.1% (w:v) SDS at 65°C and washing in 0.1x SSC containing 0.1% SDS at 65°C. Other high stringency hybridization conditions, as well as moderately stringent conditions, are described in the references cited above.

[0114] "Codon optimized" refers to changes in the codons of the polynucleotide encoding a protein to those preferentially used in a particular organism such that the encoded protein is more efficiently expressed in that organism. Although the genetic code is degenerate, in that most amino acids are represented by several codons, called "synonyms" or "synonymous" codons, it is well known that codon usage by particular organisms is nonrandom and biased towards particular codon triplets. This codon usage bias may be higher in reference to a given gene, genes of common function or ancestral origin, highly expressed proteins versus low copy number proteins, and the aggregate protein coding regions of an organism's genome. In some embodiments, the polynucleotides encoding the PAL enzymes are codon optimized for optimal production from the host organism selected for expression.

[0115] "Control sequence" refers herein to include all components, that are necessary or advantageous for the expression of a polynucleotide and/or polypeptide of the present disclosure. Each control sequence may be native or foreign to the nucleic acid sequence encoding the

polypeptide. Such control sequences include, but are not limited to, leaders, polyadenylation sequences, propeptide sequences, promoter sequences, signal peptide sequences, initiation sequences, and transcription terminators. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. In some embodiments, the control sequences are provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

[0116] "Operably linked" is defined herein as a configuration in which a control sequence is appropriately placed (*i.e.*, in a functional relationship) at a position relative to a polynucleotide of interest such that the control sequence directs or regulates the expression of the polynucleotide encoding a polypeptide of interest.

[0117] "Promoter sequence" refers to a nucleic acid sequence that is recognized by a host cell for expression of a polynucleotide of interest, such as a coding sequence. The promoter sequence contains transcriptional control sequences that mediate the expression of a polynucleotide of interest. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

[0118] "Suitable reaction conditions" refers to those conditions in the enzymatic conversion reaction solution (*e.g.*, ranges of enzyme loading, substrate loading, temperature, pH, buffers, co-solvents, etc.) under which a PAL polypeptide of the present disclosure is capable of converting a substrate to the desired product compound, Exemplary "suitable reaction conditions" are provided herein (See, the Examples).

[0119] "Loading", such as in "compound loading" or "enzyme loading" refers to the concentration or amount of a component in a reaction mixture at the start of the reaction. "Substrate" in the context of an enzymatic conversion reaction process refers to the compound or molecule acted on by the PAL polypeptide. "Product" in the context of an enzymatic conversion process refers to the compound or molecule resulting from the action of the PAL polypeptide on the substrate.

[0120] As used herein the term "culturing" refers to the growing of a population of microbial cells under suitable conditions using any suitable medium (e.g., liquid, gel, or solid) .

[0121] Recombinant polypeptides (e.g., PAL enzyme variants) can be produced using any suitable methods known the art. For example, there is a wide variety of different mutagenesis techniques well known to those skilled in the art. In addition, mutagenesis kits are also available from many commercial molecular biology suppliers. Methods are available to make specific substitutions at defined amino acids (site-directed), specific or random mutations in a localized region of the gene (regio-specific), or random mutagenesis over the entire gene (*e.g.*, saturation mutagenesis). Numerous suitable methods are known to those in the art to generate enzyme variants, including but not limited to site-directed mutagenesis of single-stranded DNA or double-stranded DNA using PCR, cassette mutagenesis, gene synthesis, error-prone PCR, shuffling, and chemical saturation mutagenesis, or any

other suitable method known in the art. Non-limiting examples of methods used for DNA and protein engineering are provided in the following patents: US Pat. No. 6,117,679; US Pat. No. 6,420,175; US Pat. No. 6,376,246; US Pat. No. 6,586,182; US Pat. No. 7,747,391; US Pat. No. 7,747,393; US Pat. No. 7,783,428; and US Pat. No. 8,383,346. After the variants are produced, they can be screened for any desired property (e.g., high or increased activity, or low or reduced activity, increased thermal activity, increased thermal stability, and/or acidic pH stability, etc.). In some embodiments, "recombinant PAL polypeptides" (also referred to herein as "engineered PAL polypeptides," "variant PAL enzymes," and "PAL variants") find use.

[0122] As used herein, a "vector" is a DNA construct for introducing a DNA sequence into a cell. In some embodiments, the vector is an expression vector that is operably linked to a suitable control sequence capable of effecting the expression in a suitable host of the polypeptide encoded in the DNA sequence. In some embodiments, an "expression vector" has a promoter sequence operably linked to the DNA sequence (*e.g.,* transgene) to drive expression in a host cell, and in some embodiments, also comprises a transcription terminator sequence.

[0123] As used herein, the term "expression" includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, and post-translational modification. In some embodiments, the term also encompasses secretion of the polypeptide from a cell.

[0124] As used herein, the term "produces" refers to the production of proteins and/or other compounds by cells. It is intended that the term encompass any step involved in the production of polypeptides including, but not limited to, transcription, post-transcriptional modification, translation, and post-translational modification. In some embodiments, the term also encompasses secretion of the polypeptide from a cell.

[0125] As used herein, an amino acid or nucleotide sequence (*e.g.*, a promoter sequence, signal peptide, terminator sequence, etc.) is "heterologous" to another sequence with which it is operably linked if the two sequences are not associated in nature.

[0126] As used herein, the terms "host cell" and "host strain" refer to suitable hosts for expression vectors comprising DNA provided herein (e.g., a polynucleotide sequences encoding at least one AvPAL variant). In some embodiments, the host cells are prokaryotic or eukaryotic cells that have been transformed or transfected with vectors constructed using recombinant DNA techniques as known in the art.

[0127] The term "analogue" means a polypeptide having more than 70 % sequence identity but less than 100% sequence identity (e.g., more than 75%, 78%, 80%, 83%, 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity) with a reference polypeptide. In some embodiments, analogues include non-naturally occurring amino acid residues including, but not limited, to homoarginine, ornithine and norvaline, as well as naturally occurring amino acids. In some

embodiments, analogues also include one or more D-amino acid residues and non-peptide linkages between two or more amino acid residues.

[0128] The term "therapeutic" refers to a compound administered to a subject who shows signs or symptoms of pathology having beneficial or desirable medical effects.

[0129] The term "pharmaceutical composition" refers to a composition suitable for pharmaceutical use in a mammalian subject (e.g., human) comprising a pharmaceutically effective amount of an engineered PAL polypeptide encompassed by the invention and an acceptable carrier.

[0130] The term "effective amount" means an amount sufficient to produce the desired result. One of general skill in the art may determine what the effective amount by using routine experimentation.

[0131] The terms "isolated" and "purified" are used to refer to a molecule (e.g., an isolated nucleic acid, polypeptide, etc.) or other component that is removed from at least one other component with which it is naturally associated. The term "purified" does not require absolute purity, rather it is intended as a relative definition.

[0132] The term "subject" encompasses mammals such as humans, non-human primates, livestock, companion animals, and laboratory animals (e.g., rodents and lagamorphs). It is intended that the term encompass females as well as males.

[0133] As used herein, the term "patient" means any subject that is being assessed for, treated for, or is experiencing disease.

[0134] The term "infant" refers to a child in the period of the first month after birth to approximately one (1) year of age. As used herein, the term "newborn" refers to child in the period from birth to the 28^{th} day of life. The term "premature infant" refers to an infant born after the twentieth completed week of gestation, yet before full term, generally weighing ~500 to ~2499 grams at birth. A "very low birth weight infant" is an infant weighing less than 1500 g at birth.

[0135] As used herein, the term "child" refers to a person who has not attained the legal age for consent to treatment or research procedures. In some embodiments, the term refers to a person between the time of birth and adolescence.

[0136] As used herein, the term "adult" refers to a person who has attained legal age for the relevant jurisdiction (e.g., 18 years of age in the United States). In some embodiments, the term refers to any fully grown, mature organism. In some embodiments, the term "young adult" refers to a person less than 18 years of age, but who has reached sexual maturity.

[0137] As used herein, "composition" and "formulation" encompass products comprising at least one engineered PAL of the present invention, intended for any suitable use (e.g., pharmaceutical compositions, dietary/nutritional supplements, feed, etc.).

[0138] The terms "administration" and "administering" a composition mean providing a composition of the present invention to a subject (e.g., to a person suffering from the effects of PKU).

[0139] The term "carrier" when used in reference to a pharmaceutical composition means any of the standard pharmaceutical carrier, buffers, and excipients, such as stabilizers, preservatives, and adjuvants.

[0140] The term "pharmaceutically acceptable" means a material that can be administered to a subject without causing any undesirable biological effects or interacting in a deleterious manner with any of the components in which it is contained and that possesses the desired biological activity.

[0141] As used herein, the term "excipient" refers to any pharmaceutically acceptable additive, carrier, diluent, adjuvant, or other ingredient, other than the active pharmaceutical ingredient (API; e.g., the engineered PAL polypeptides of the present invention). Excipients are typically included for formulation and/or administration purposes.

[0142] The term "therapeutically effective amount" when used in reference to symptoms of disease/condition refers to the amount and/or concentration of a compound (e.g., engineered PAL polypeptides) that ameliorates, attenuates, or eliminates one or more symptom of a disease/condition or prevents or delays the onset of symptom(s) (e.g., PKU). In some embodiments, the term is use in reference to the amount of a composition that elicits the biological (e.g., medical) response by a tissue, system, or animal subject that is sought by the researcher, physician, veterinarian, or other clinician.

[0143] The term "therapeutically effective amount" when used in reference to a disease/condition refers to the amount and/or concentration of a composition that ameliorates, attenuates, or eliminates the disease/condition.

[0144] It is intended that the terms "treating," "treat" and "treatment" encompass preventative (e.g., prophylactic), as well as palliative treatment.

Engineered PAL Polypeptides:

[0145] The parent PAL polypeptides from which the engineered PAL polypeptides of the invention are derived from include bacterial strains such as *Anabaena* (e.g., *A. variabilis*), *Nostoc* (e.g., *N. punctiforme*), *Rhodosporidium* (e.g., *R. toruloides*), *Streptomyces* (e.g., *S. maritimus or S. verticillatus*), *Oscillatoria* sp., *Gloeocapsa* sp., and *Rivularia* sp. PAL enzymes from these strains have been identified and are well known. Homologous enzyme sequences from *Anabaena* (*A. variabilis*) ATCC 29413 and NCBI YP_324488.1; *Nostoc* (*N. punctiforme*) ATCC 29133 and NCBI YP_00186563.1; *Oscillatoria* sp. PCC 6506 and NCBI ZP_ 07108482.1 and *Gloeocapsa* sp. PCC7428 and NCBI YP_007127054.1 are provided in Figure 1. The *Nostoc punctiforme* phenylalanine/histidine ammonia lyase "NpPHAL" (NCBI YP_001865631.1 (SEQ ID NO:30); *Rivularia sp.* histidine ammonia-lyase "RspHAL" (NCBI YP_007056096.1 (SEQ ID NO:31); *Oscillatoria sp.* histidine ammonia-lyase "Osp HAL" (NCBI YP_07108482.1(SEQ ID NO:32); and *Gloeocapsa sp.* histidine ammonia-lyase "GspHAL" (NCBI YP_007127054.1) (SEQ ID NO:33) have more than 70% homology with AvPAL (SEQ ID NO:4).

[0146] Furthermore, when a particular PAL variant (i.e., an engineered PAL polypeptide) is referred to by reference to modification of particular amino acids residues in the sequence of a wild-type PAL or reference PAL it is to be understood that variants of another PAL modified in the equivalent position(s) (as determined from the optional amino acid sequence alignment between the respective amino acid sequences) are encompassed herein. In some embodiments, the engineered PAL polypeptide is derived from any one of the polypeptides listed from the bacterial strains above (i.e., *Nostoc* [*N. punctiforme*], *Rhodosporidium* [*R. toruloides*], *Streptomyces* [*S. maritimus or S. verticillatus*], *Oscillatoria* sp. , *Gloeocapsa* sp and *Rivularia* sp.). In some additional embodiments, the engineered PAL polypeptide of the present invention comprises the conserved active site Ala167-Ser168-Gly169 and comprises at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO:4. In some embodiments, the engineered PAL polypeptides comprise not only PAL activity but are also active on tyrosine and/or histidine substrates.

[0147] In some embodiments, engineered PAL polypeptides are produced by cultivating a microorganism comprising at least one polynucleotide sequence encoding at least one engineered PAL polypeptide under conditions which are conducive for producing the engineered PAL polypeptide. In some embodiments, the engineered PAL polypeptide is subsequently recovered from the resulting culture medium and/or cells.

[0148] The present invention provides exemplary engineered PAL polypeptides having PAL activity. The Examples provide Tables showing sequence structural information correlating specific amino acid sequence features with the functional activity of the engineered PAL polypeptides. This structure-function correlation information is provided in the form of specific amino acid residue differences relative to the reference engineered polypeptide of SEQ ID NO:4, as well as associated experimentally determined activity data for the exemplary engineered PAL polypeptides.

[0149] In some embodiments, the engineered PAL polypeptides of the present invention having PAL activity comprise a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:4; b) an amino acid residue difference as compared to SEQ ID NO:4 at one or more amino acid positions; and c) which exhibits an improved property selected from i) enhanced catalytic activity, ii) reduced proteolytic sensitivity, iii) increased tolerance to acidic pH, iv) reduced aggregation or a combination of any of i), ii), iii) or iv) as compared to the reference sequence.

[0150] In some embodiments the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4, and an amino acid residue difference as compared to SEQ ID NO:4, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 20 or more amino acid positions compared to SEQ ID NO:4 or a sequence having at least 85%, at least

88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4). In some embodiments, the residue difference as compared to SEQ ID NO:4, at one or more positions includes at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more conservative amino acid substitutions. In some embodiments, the engineered PAL polypeptide is a polypeptide listed in the Tables provided in the Examples.

[0151] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4 and an amino acid residue difference as compared to SEQ ID NO:4 at one or more amino acid positions are selected from X39; X54; X59; X73; X91; X158; X112, X134, X180; X195; X240; X243; X245; X256; X257; X270; X290; X304, X305; X307; X308; X326; X349; X353; X364; X394; X399; X400; X404; X407; X443; X453; X459; X460; X463; X474; X509; X521; X522; X524; X528; X546; X564; or any combination thereof, when optimally aligned with the amino acid sequence of SEQ ID NO:4. In some embodiments the amino acid difference is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 or greater amino acid positions.

[0152] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85% (at least 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) sequence identity to SEQ ID NO:4 and comprise an amino acid residue difference at position H307 and optionally an amino acid residue difference at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments the amino acid residue difference at position 307 is H307/G/Q/M.

[0153] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85% (at least 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) sequence identity to SEQ ID NO:4 and comprise at least one amino acid residue difference selected from a combination of one or more of A39; T54; G59, S73; A91; Y158; S180; K195; A112; R134; Q240; T243; I245; A256; L257; N270; N290; Y304; R305; H307; E308; I326; L349; D353; L364; A394; S399; N400; P404; L407; F443; N453; Y459; T460; T463; N474; E509; Q521; K522; T524; P528; S546; and/or P564. In some additional embodiments, there are amino acid residue differences at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions.

[0154] In some embodiments, the engineered PAL polypeptides exhibiting an improved property have at least 85% (at least 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%) sequence identity to SEQ ID NO:4 and comprise an amino acid residue difference selected from a combination of one or more of A39V; T54K; G59R; S73K; A112C; R134Q; A91V; Y158H; S180A; K195E; Q240R/W; T243I/L; I245L; A256G; L257W/A; N270K; N290G; Y304H; R305M; H307G/Q/M; E308Q; I326F; L349M; D353A/N; L364Q; A394V; S399N; N400K; P404A; L407V; F443H; N453G; Y459F; T460G; T463N; N474Q; E509L; Q521K/S; K522Y/F/N; T524S;P528L; S546R; and P564 G/L/M, when optimally aligned with SEQ ID NO:4.

[0155] In some embodiments, the amino acid residue difference is selected from a combination of one or more of A39V; A91V; A256G; N290G; A394V; S399N; P404A; L407V; K522Y/F/N; and/or T524S, when optimally aligned with SEQ ID NO:4.

[0156] In some embodiments, the present invention provides functional fragments of engineered PAL polypeptides. In some embodiments, functional fragments comprise at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% of the activity of the engineered PAL polypeptide from which it was derived (i.e., the parent engineered PAL). In some embodiments, functional fragments comprise at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% of the parent sequence of the engineered PAL. In some embodiments the functional fragment will be truncated by less than 5, less than 10, less than 15, less than 10, less than 25, less than 30, less than 35, less than 40, less than 45, and less than 50 amino acids.

[0157] In some embodiments, the present invention provides functional fragments of engineered PAL polypeptides. In some embodiments, functional fragments comprise at least about 95%, 96%, 97%, 98%, or 99% of the activity of the engineered PAL polypeptide from which it was derived (i.e., the parent engineered PAL). In some embodiments, functional fragments comprise at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the parent sequence of the engineered PAL. In some embodiments the functional fragment will be truncated by less than 5, less than 10, less than 15, less than 10, less than 25, less than 30, less than 35, less than 40, less than 45, less than 50, less than 55, less than 60, less than 65, or less than 70 amino acids.

[0158] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property has at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:6 and an amino acid residue difference as compared to SEQ ID NO:6, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:6, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:6. In some embodiments, the engineered PALs comprise at least 90% sequence identity to SEQ ID NO:6 and comprise an amino acid difference as compared to SEQ ID NO:6 of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:6.

[0159] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:10, or a functional fragment thereof and an amino acid residue

difference as compared to SEQ ID NO:10, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:10, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:10. In some embodiments, the engineered PALs comprise at least 95% sequence identity to SEQ ID NO:10, and comprise an amino acid difference as compared to SEQ ID NO:10, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:10.

[0160] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:12 or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:12 at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:12, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:12. In some embodiments, the engineered PALs comprise at least 95% sequence identity to SEQ ID NO:12, and comprise an amino acid difference as compared to SEQ ID NO:12, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:12.

[0161] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:14 or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:14 at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:14, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:14. In some embodiments, the engineered PALs comprise at least 95% sequence identity to SEQ ID NO:14, and comprise an amino acid difference as compared to SEQ ID NO:14, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:14.

[0162] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:16 or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:16 at one or more amino acid positions (such as at 1, 2, 3, 4, 5,

6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:16, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:16. In some embodiments, the engineered PALs comprise at least 95% sequence identity to SEQ ID NO:16, and comprise an amino acid difference as compared to SEQ ID NO:16, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:16.

[0163] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:18 or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:18 at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:18, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:18. In some embodiments, the engineered PALs comprise at least 95% sequence identity to SEQ ID NO:18, and comprise an amino acid difference as compared to SEQ ID NO:18, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:18.

[0164] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:20 or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:20 at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:20, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:20. In some embodiments, the engineered PALs comprise at least 95% sequence identity to SEQ ID NO:20, and comprise an amino acid difference as compared to SEQ ID NO:20, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:20.

[0165] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:22 or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:22 at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:22, or a sequence

having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:22. In some embodiments, the engineered PALs comprise at least 95% sequence identity to SEQ ID NO:22, and comprise an amino acid difference as compared to SEQ ID NO:22, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:22.

[0166] In some embodiments, the engineered PAL polypeptides exhibiting at least one improved property have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:24 or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NO:24 at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NO:24, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:24. In some embodiments, the engineered PALs comprise at least 95% sequence identity to SEQ ID NO:24, and comprise an amino acid difference as compared to SEQ ID NO:24, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the engineered PAL polypeptide consists of the sequence of SEQ ID NO:24.

Variants with Reduced Sensitivity to Proteolysis:

[0167] In some embodiments, the engineered PAL polypeptides of the present invention have PAL activity, exhibit reduced sensitivity to proteolysis, and comprise: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:4; an b) an amino acid residue difference as compared to SEQ ID NO:4 at one or more amino acid positions.

[0168] In some embodiments, the engineered PAL polypeptides that exhibit reduced sensitivity to proteolysis have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4 and an amino acid residue difference as compared to SEQ ID NO:4 at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 20 or more amino acid positions compared to SEQ ID NO:4 or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NO:4).

[0169] In some embodiments, the engineered PAL polypeptides that exhibit reduced sensitivity to proteolysis have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4 and an amino acid residue difference as compared to SEQ ID NO:4, at one or more amino acid positions are selected from X39; X54; X59; X73; X91; X158; X112,

X134, X180; X195; X240; X243; X245; X256; X257; X270; X290; X304, X305; X307; X308; X326; X349; X353; X364; X394; X399; X400; X404; X407; X443; X453; X459; X460; X463; X474; X509; X521; X522; X524; X528; X546; X564; or any combination thereof, when optimally aligned with the amino acid sequence of SEQ ID NO: 4. In some embodiments the amino acid difference is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 or greater amino acid positions.

[0170] In some embodiments, the engineered PAL polypeptides that exhibit reduced sensitivity to proteolysis have at least 85%, at least 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:4, and comprise an amino acid residue difference at position X307; X326; X460; X307; and/or X528 and optionally an amino acid residue difference at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the amino acid residue difference is selected from Y304H/W; R305L/M; H307G/M/Q; I326F; Q240W; T460G; P528L; and any of these substitutions in combination, when aligned with SEQ ID NO:4.

[0171] In some embodiments, the engineered PAL polypeptides that exhibit reduced sensitivity to proteolysis have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with any of SEQ ID NOS:10, 12, 14, 16, 18, 20, 22, and/or 24, or a functional fragment thereof and an amino acid residue difference as compared to SEQ ID NOS:10, 12, 14, 16, 18, 20, 22, and/or 24, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15 or more amino acid positions) compared to SEQ ID NOS:10, 12, 14, 16, 18, 20, 22, and/or 24, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or greater amino acid sequence identity with SEQ ID NOS:10, 12, 14, 16, 18, 20, 22, and/or 24. In some embodiments, the engineered PAL comprises at least 95% sequence identity to SEQ ID NOS:10, 12, 14, 16, 18, 20, 22, and/or 24, and comprise an amino acid difference as compared to SEQ ID NOS:10, 12, 14, 16, 18, 20, 22, and/or 24, of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments, the PAL comprises or consists of the sequence of SEQ ID NO:10, 12, 14, 16, 18, 20, 22, and/or 24.

[0172] In some embodiments, the proteolytic sensitivity of the engineered PAL polypeptides is reduced by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95%, of that of the wild-type PAL (e.g., AvPAL having SEQ ID NO:4) or as compared to a reference PAL polypeptide under essentially the same conditions. The proteolytic activity can be measured using any suitable methods known in the art, including but not limited to those described in the Examples.

[0173] In some embodiments, the engineered PAL polypeptides having reduced sensitivity to proteolysis have reduced sensitivity to a composition comprising one or more proteases, including, but not limited to pepsin, trypsin, chymotrypsin, carboxypeptidase A and B, peptidases (e.g., amino peptidase, dipeptidase and enteropeptidase) when both the reference PAL and the engineered PAL

having the reduced sensitivity are compared and exposed to essentially the same amount and kind of protease under essentially the same conditions.

[0174] In some embodiments, the engineered PAL polypeptide having reduced sensitivity to proteolysis have enzyme activity levels that are about 1.0 fold, 2-fold, 5-fold, 10-fold, 20-fold, 25-fold, 50-fold, 75-fold, 100-fold, 150-fold, 200-fold or more of the enzymatic activity of the reference PAL (e.g., AvPAL). In some embodiments, the engineered polypeptides have more enzyme activity, as compared to a reference PAL, when activity is measured at a pH range of 4.5 to 7.5; when activity is measured at a pH range of 4.5 to 6.5; when activity is measured at a pH range of 5.0 to 7.5; when activity is measured at a pH range of 5.0 to 6.5; when activity is measured at a pH range of 5.5 to 7.5; and/or also when activity is measured at a pH range of 5.5 to 6.5. In some other embodiments, the engineered PAL polypeptides have K_m values in the range of 1μM to 5mM.

Variants with Increased Tolerance to Acidic pH:

[0175] In some embodiments, the engineered PAL polypeptides of the invention have PAL activity, are tolerant to acidic pH levels and comprise: a) an amino acid sequence having at least 85% sequence identity to reference sequence SEQ ID NO:4, or a fragment thereof; and b) an amino acid residue difference as compared to SEQ ID NO:4, at one or more amino acid positions.

[0176] In some embodiments, the engineered PAL polypeptides that exhibit increased tolerance to acidic pH as compared to wild-type AvPAL and/or another reference polypeptide have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4 and an amino acid residue difference as compared to SEQ ID NO:4, at one or more amino acid positions (such as at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 20 or more amino acid positions compared to SEQ ID NO:4, or a sequence having at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4.

[0177] In some embodiments, the engineered PAL polypeptides that exhibit increased tolerance to acidic pH as compared to wild-type AvPAL and/or another reference polypeptide, have at least 85%, at least 88%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater amino acid sequence identity with SEQ ID NO:4, and an amino acid residue difference as compared to SEQ ID NO:4, at one or more amino acid positions are selected from X39; X54; X59; X73; X91; X158; X112, X134, X180; X195; X240; X243; X245; X256; X257; X270; X290; X304, X305; X307; X308; X326; X349; X353; X364; X394; X399; X400; X404; X407; X443; X453; X459; X460; X463; X474; X509; X521; X522; X524; X528; X546; X564; or any combination thereof when optimally aligned with the amino acid sequence of SEQ ID NO:4. In some embodiments the amino acid difference is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 or greater amino acid positions.

[0178] In some embodiments, the engineered PAL polypeptides that exhibit increased tolerance to acidic pH as compared to wild-type AvPAL and/or another reference polypeptide have at least 85%, at least 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:4, and comprise an amino acid residue difference at position X39; X54; X59; X73; X91; X158; X112, X134, X180; X195; X240; X243; X245; X256; X257; X270; X290; X304, X305; X307; X308; X326; X349; X353; X364; X394; X399; X400; X404; X407; X443; X453; X459; X460; X463; X474; X509; X521; X522; X524; X528; X546; X564; or any combination thereof; and optionally an amino acid residue difference at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid positions. In some embodiments the amino acid residue difference is A39; T54; G59, S73; A91; Y158; S180; K195; A112; R134; Q240; T243; I245; A256; L257; N270; N290; Y304; R305; H307; E308; I326; L349; D353; L364; A394; S399; N400; P404; L407; F443; N453; Y459; T460; T463; N474; E509; Q521; K522; T524; P528; S546; and/or P564, when aligned with SEQ ID NO:4. In some embodiments, the engineered PAL polypeptides that exhibit increased tolerance to acidic pH have at least 85%, at least 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:4, and comprise an amino acid residue difference at one or more positions A39V; T54K; G59R; S73K; A112C; R134Q; A91V; Y158H; S180A; K195E; Q240R/W; T243I/L; I245L; A256G; L257W/A; N270K; N290G; Y304H; R305M; H307G/Q/M; E308Q; I326F; L349M; D353A/N; L364Q; A394V; S399N; N400K; P404A; L407V; F443H; N453G; Y459F; T460G; T463N; N474Q; E509L; Q521K/S; K522Y/F/N; T524S;P528L; S546R; and/or P564 G/L/M; when aligned with SEQ ID NO:4.

[0179] In some embodiments, when all other assay conditions are essentially the same, the engineered PAL polypeptides having increased tolerance to acidic pH as compared to a reference PAL polypeptide have an increased tolerance at a pH range between 1.5 to 6.5; between 1.5 and 5.0; between 2.0 to 5.5; between 3.0 and 6.8; between 3.0 and 5.5; between 4.0 and 6.5; between 4.0 and 4.5; between 4.5 and between 5.0; between 4.5 and 5.5, between 4.5 and 6.0; between 4.5 and 6.5; between 5.0 and 6.5; between 5.0 and 6.0; between 5.0 and 5.5; between 5.5 and 6.0; between 6.0 and 6.5; and/or between 6.5 and 7.0. In some embodiments, the increased tolerance to acidic pH is exhibited at a pH of about 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and/or 6.5.

[0180] In some embodiments, the engineered PAL polypeptides that have increased tolerance to acidic pH also exhibit greater PAL activity as compared to a reference PAL when measure by a standard assay. Any suitable assay finds use in the present invention, including, but not limited to those provided herein.

[0181] It is further contemplated that any of the exemplary engineered polypeptides (i.e., Variant No. 1 – Variant No. 1010) find use as the starting amino acid sequence for synthesizing other engineered PAL polypeptides, for example by subsequent rounds of evolution by adding new combinations of various amino acid differences from other polypeptides and other residue positions described herein. In some embodiments, additional improvements are generated by including amino acid differences at residue positions that were maintained as unchanged throughout earlier rounds of evolution. It is not

intended that the present invention be limited to any particular method for producing engineered PAL polypeptides, as any suitable method finds use, including but not limited to the methods provided herein.

Polynucleotides Encoding Engineered Polypeptides, Expression Vectors and Host Cells:

[0182] The present invention provides polynucleotides encoding the engineered PAL polypeptides described herein. In some embodiments, the polynucleotides are operatively linked to one or more heterologous regulatory sequences that control gene expression to create a recombinant polynucleotide capable of expressing the polypeptide. In some embodiments, expression constructs containing at least one heterologous polynucleotide encoding the engineered PAL polypeptide(s) is introduced into appropriate host cells to express the corresponding PAL polypeptide(s).

[0183] As will be apparent to the skilled artisan, availability of a protein sequence and the knowledge of the codons corresponding to the various amino acids provide a description of all the polynucleotides capable of encoding the subject polypeptides. The degeneracy of the genetic code, where the same amino acids are encoded by alternative or synonymous codons, allows an extremely large number of nucleic acids to be made, all of which encode an engineered PAL polypeptide. Thus, the present invention provides methods and compositions for the production of each and every possible variation of PAL polynucleotides that could be made that encode the PAL polypeptides described herein by selecting combinations based on the possible codon choices, and all such variations are to be considered specifically disclosed for any polypeptide described herein, including the amino acid sequences presented in the Examples (e.g., in the various Tables) .

[0184] In some embodiments, the codons are preferably optimized for utilization by the chosen host cell for protein production. For example, preferred codons used in bacteria are typically used for expression in bacteria. Consequently, codon optimized polynucleotides encoding the engineered PAL polypeptides contain preferred codons at about 40%, 50%, 60%, 70%, 80%, 90%, or greater than 90% of the codon positions in the full length coding region.

[0185] In some embodiments, the PAL polynucleotide encodes an engineered polypeptide having PAL activity with the properties disclosed herein, wherein the polypeptide comprises an amino acid sequence having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to a reference sequence selected from SEQ ID NOS:3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and/or 23, or the amino acid sequence of any variant (e.g., those provided in the Examples), and one or more residue differences as compared to the reference polynucleotide of SEQ ID NOs:3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and/or 23, or the amino acid sequence of any variant as disclosed in the Examples (for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid residue positions). In some embodiments, the reference sequence is selected from SEQ ID NOS:3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and/or 23.

[0186] In some embodiments, the PAL polynucleotide encodes an engineered polypeptide having PAL activity with the properties disclosed herein, wherein the polypeptide comprises an amino acid sequence having at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to reference sequence SEQ ID NO:4 and one or more residue differences as compared to SEQ ID NO:4 at residue positions from X39; X54; X59; X73; X91; X158; X112, X134, X180; X195; X240; X243; X245; X256; X257; X270; X290; X304, X305; X307; X308; X326; X349; X353; X364; X394; X399; X400; X404; X407; X443; X453; X459; X460; X463; X474; X509; X521; X522; X524; X528; X546; and/or X564; when optimally aligned with the polypeptide of SEQ ID NO:4.

[0187] In some embodiments, the polynucleotide encoding the engineered PAL polypeptides comprises a polynucleotide sequence selected from a polynucleotide sequence selected from SEQ ID NOS:3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and/or 23. In some embodiments, the polynucleotide encoding an engineered PAL polypeptide has at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 99% nucleotide residue identity to SEQ ID NOS:2, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and/or 23.

[0188] In some embodiments, the polynucleotides are capable of hybridizing under highly stringent conditions to a reference polynucleotide sequence selected from SEQ ID NOS:2, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and/or 23, or a complement thereof, or a polynucleotide sequence encoding any of the variant PAL polypeptides provided herein . In some embodiments, the polynucleotide capable of hybridizing under highly stringent conditions encodes a PAL polypeptide comprising an amino acid sequence that has one or more residue differences as compared to SEQ ID NO:4, at residue positions selected from X39; X54; X59; X73; X91; X158; X112, X134, X180; X195; X240; X243; X245; X256; X257; X270; X290; X304, X305; X307; X308; X326; X349; X353; X364; X394; X399; X400; X404; X407; X443; X453; X459; X460; X463; X474; X509; X521; X522; X524; X528; X546; and/or X564.

[0189] In some embodiments, an isolated polynucleotide encoding any of the engineered PAL polypeptides herein is manipulated in a variety of ways to facilitate expression of the PAL polypeptide. In some embodiments, the polynucleotides encoding the PAL polypeptides comprise expression vectors where one or more control sequences is present to regulate the expression of the PAL polynucleotides and/or polypeptides. Manipulation of the isolated polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector utilized. Techniques for modifying polynucleotides and nucleic acid sequences utilizing recombinant DNA methods are well known in the art. In some embodiments, the control sequences include among others, promoters, leader sequences, polyadenylation sequences, propeptide sequences, signal peptide sequences, and transcription terminators. In some embodiments, suitable promoters are selected based on the host cells selection. For bacterial host cells, suitable promoters for directing transcription of the nucleic acid constructs of the present disclosure, include, but are not limited to promoters obtained from the *E. coli* lac operon, *Streptomyces coelicolor* agarase gene (dagA), *Bacillus subtilis*

levansucrase gene (sacB), *Bacillus licheniformis* alpha-amylase gene (amyL), *Bacillus stearothermophilus* maltogenic amylase gene (amyM), *Bacillus amyloliquefaciens* alpha-amylase gene (amyQ), *Bacillus licheniformis* penicillinase gene (penP), *Bacillus subtilis* xylA and xylB genes, and prokaryotic beta-lactamase gene (See e.g., Villa-Kamaroff et al., Proc. Natl Acad. Sci. USA 75: 3727-3731 [1978]), as well as the *tac* promoter (See e.g., DeBoer et al., Proc. Natl Acad. Sci. USA 80: 21-25 [1983]). Exemplary promoters for filamentous fungal host cells, include, but are not limited to promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (glaA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, and *Fusarium oxysporum* trypsin-like protease (See e.g., WO 96/00787), as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase), and mutant, truncated, and hybrid promoters thereof. Exemplary yeast cell promoters can be from the genes can be from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are known in the art (See e.g., Romanos et al., Yeast 8:423-488 [1992]).

[0190] In some embodiments, the control sequence is also a suitable transcription terminator sequence (i.e., a sequence recognized by a host cell to terminate transcription). In some embodiments, the terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the PAL polypeptide. Any suitable terminator which is functional in the host cell of choice finds use in the present invention. Exemplary transcription terminators for filamentous fungal host cells can be obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease. Exemplary terminators for yeast host cells can be obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are known in the art (See e.g., Romanos et al., *supra*).

[0191] In some embodiments, the control sequence is also a suitable leader sequence(i.e., a non-translated region of an mRNA that is important for translation by the host cell). in some embodiments, the leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the PAL polypeptide. Any suitable leader sequence that is functional in the host cell of choice find use in the present invention. Exemplary leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, and *Aspergillus nidulans* triose phosphate isomerase. Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and

Saccharomyces cerevisiae alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

[0192] In some embodiments, the control sequence is also a polyadenylation sequence (i.e., a sequence operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA). Any suitable polyadenylation sequence which is functional in the host cell of choice finds use in the present invention. Exemplary polyadenylation sequences for filamentous fungal host cells include, but are not limited to the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease, and *Aspergillus niger* alpha-glucosidase. Useful polyadenylation sequences for yeast host cells are known (See e.g., Guo and Sherman, Mol. Cell. Bio., 15:5983-5990 [1995]).

[0193] In some embodiments, the control sequence is also a signal peptide (i.e., a coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway). In some embodiments, the 5' end of the coding sequence of the nucleic acid sequence inherently contains a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the secreted polypeptide. Alternatively, in some embodiments, the 5' end of the coding sequence contains a signal peptide coding region that is foreign to the coding sequence. Any suitable signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice finds use for expression of the engineered polypeptide(s). Effective signal peptide coding regions for bacterial host cells are the signal peptide coding regions include, but are not limited to those obtained from the genes for *Bacillus* NClB 11837 maltogenic amylase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* neutral proteases (nprT, nprS, nprM), and *Bacillus subtilis* prsA. Further signal peptides are known in the art (See e.g., Simonen and Palva, Microbiol. Rev., 57:109-137 [1993]). In some embodiments, effective signal peptide coding regions for filamentous fungal host cells include, but are not limited to the signal peptide coding regions obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, and *Humicola lanuginosa* lipase. Useful signal peptides for yeast host cells include, but are not limited to those from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase.

[0194] In some embodiments, the control sequence is also a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is referred to as a "proenzyme," "propolypeptide," or "zymogen." A propolypeptide can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from any suitable source, including, but not limited to the genes for *Bacillus subtilis* alkaline protease (aprE), *Bacillus subtilis* neutral

protease (nprT), *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* lactase (See e.g., WO 95/33836). Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

[0195] In some embodiments, regulatory sequences are also utilized. These sequences facilitate the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those that cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. In prokaryotic host cells, suitable regulatory sequences include, but are not limited to the *lac*, *tac*, and *trp* operator systems. In yeast host cells, suitable regulatory systems include, but are not limited to the ADH2 system or GAL1 system. In filamentous fungi, suitable regulatory sequences include, but are not limited to the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter.

[0196] In another aspect, the present invention is directed to a recombinant expression vector comprising a polynucleotide encoding an engineered PAL polypeptide, and one or more expression regulating regions such as a promoter and a terminator, a replication origin, etc., depending on the type of hosts into which they are to be introduced. In some embodiments, the various nucleic acid and control sequences described herein are joined together to produce recombinant expression vectors which include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the PAL polypeptide at such sites. Alternatively, in some embodiments, the nucleic acid sequence of the present invention is expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In some embodiments involving the creation of the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

[0197] The recombinant expression vector may be any suitable vector (*e.g.*, a plasmid or virus), that can be conveniently subjected to recombinant DNA procedures and bring about the expression of the PAL polynucleotide sequence. The choice of the vector typically depends on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

[0198] In some embodiments, the expression vector is an autonomously replicating vector (i.e., a vector that exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid, an extra-chromosomal element, a minichromosome, or an artificial chromosome). The vector may contain any means for assuring self-replication. In some alternative embodiments, the vector is one in which, when introduced into the host cell, it is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated.

Furthermore, in some embodiments, a single vector or plasmid, or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, and/or a transposon is utilized.

[0199] In some embodiments, the expression vector contains one or more selectable markers, which permit easy selection of transformed cells. A "selectable marker" is a gene, the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers include, but are not limited to the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers, which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Suitable markers for yeast host cells include, but are not limited to ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in filamentous fungal host cells include, but are not limited to, amdS (acetamidase; e.g., from *A. nidulans* or *A. orzyae*), argB (ornithine carbamoyltransferases), bar (phosphinothricin acetyltransferase; e.g., from S. *hygroscopicus*), hph (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase; e.g., from *A. nidulans* or *A. orzyae*), sC (sulfate adenyltransferase), and trpC (anthranilate synthase), as well as equivalents thereof. In another aspect, the present invention provides a host cell comprising at least one polynucleotide encoding at least one engineered PAL polypeptide of the present invention, the polynucleotide(s) being operatively linked to one or more control sequences for expression of the engineered PAL enzyme(s) in the host cell. Host cells suitable for use in expressing the polypeptides encoded by the expression vectors of the present invention are well known in the art and include but are not limited to, bacterial cells, such as *E. coli*, *Vibrio fluvialis*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells (*e.g.*, *Saccharomyces cerevisiae* or *Pichia pastoris* (ATCC Accession No. 201178)); insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, BHK, 293, and Bowes melanoma cells; and plant cells. Exemplary host cells also include various *Escherichia coli* strains (e.g., W3110 (ΔfhuA) and BL21).

[0200] Accordingly, in another aspect, the present invention provides methods of producing the engineered PAL polypeptides, where the methods comprise culturing a host cell capable of expressing a polynucleotide encoding the engineered PAL polypeptide under conditions suitable for expression of the polypeptide. In some embodiments, the methods further comprise the steps of isolating and/or purifying the PAL polypeptides, as described herein.

[0201] Appropriate culture media and growth conditions for host cells are well known in the art. It is contemplated that any suitable method for introducing polynucleotides for expression of the PAL polypeptides into cells will find use in the present invention. Suitable techniques include, but are not limited to electroporation, biolistic particle bombardment, liposome mediated transfection, calcium chloride transfection, and protoplast fusion.

[0202] Engineered PAL polypeptides with the properties disclosed herein can be obtained by subjecting the polynucleotide encoding the naturally occurring or engineered PAL polypeptide to any

suitable mutagenesis and/or directed evolution methods known in the art, and/or as described herein. An exemplary directed evolution technique is mutagenesis and/or DNA shuffling (See e.g., Stemmer, Proc. Natl. Acad. Sci. USA 91:10747-10751 [1994]; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767 and U.S. Pat. 6,537,746). Other directed evolution procedures that can be used include, among others, staggered extension process (StEP), in vitro recombination (See e.g., Zhao et al., Nat. Biotechnol., 16:258–261 [1998]), mutagenic PCR (See e.g., Caldwell et al., PCR Methods Appl., 3:S136-S140 [1994]), and cassette mutagenesis (See e.g., Black et al., Proc. Natl. Acad. Sci. USA 93:3525-3529 [1996]).

[0203] Mutagenesis and directed evolution methods can be readily applied to PAL-encoding polynucleotides to generate variant libraries that can be expressed, screened, and assayed. Any suitable mutagenesis and directed evolution methods find use in the present invention and are well known in the art (*See e.g.,* US Patent Nos. 5,605,793, 5,830,721, 6,132,970, 6,420,175, 6,277,638, 6,365,408, 6,602,986, 7,288,375, 6,287,861, 6,297,053, 6,576,467, 6,444,468, 5,811238, 6,117,679, 6,165,793, 6,180,406, 6,291,242, 6,995,017, 6,395,547, 6,506,602, 6,519,065, 6,506,603, 6,413,774, 6,573,098, 6,323,030, 6,344,356, 6,372,497, 7,868,138, 5,834,252, 5,928,905, 6,489,146, 6,096,548, 6,387,702, 6,391,552, 6,358,742, 6,482,647, 6,335,160, 6,653,072, 6,355,484, 6,03,344, 6,319,713, 6,613,514, 6,455,253, 6,579,678, 6,586,182, 6,406,855, 6,946,296, 7,534,564, 7,776,598, 5,837,458, 6,391,640, 6,309,883, 7,105,297, 7,795,030, 6,326,204, 6,251,674, 6,716,631, 6,528,311, 6,287,862, 6,335,198, 6,352,859, 6,379,964, 7,148,054, 7,629,170, 7,620,500, 6,365,377, 6,358,740, 6,406,910, 6,413,745, 6,436,675, 6,961,664, 7,430,477, 7,873,499, 7,702,464, 7,783,428, 7,747,391, 7,747,393, 7,751,986, 6,376,246, 6,426,224, 6,423,542, 6,479,652, 6,319,714, 6,521,453, 6,368,861, 7,421,347, 7,058,515, 7,024,312, 7,620,502, 7,853,410, 7,957,912, 7,904,249, and all related non-US counterparts; Ling et al., Anal. Biochem., 254(2):157-78 [1997]; Dale et al., Meth. Mol. Biol., 57:369-74 [1996]; Smith, Ann. Rev. Genet., 19:423-462 [1985]; Botstein et al., Science, 229:1193-1201 [1985]; Carter, Biochem. J., 237:1-7 [1986]; Kramer et al., Cell, 38:879-887 [1984]; Wells et al., Gene, 34:315-323 [1985]; Minshull et al., Curr. Op. Chem. Biol., 3:284-290 [1999]; Christians et al., Nat. Biotechnol., 17:259-264 [1999]; Crameri et al., Nature, 391:288-291 [1998]; Crameri, et al., Nat. Biotechnol., 15:436-438 [1997]; Zhang et al., Proc. Nat. Acad. Sci. U.S.A., 94:4504-4509 [1997]; Crameri et al., Nat. Biotechnol., 14:315-319 [1996]; Stemmer, Nature, 370:389-391 [1994]; Stemmer, Proc. Nat. Acad. Sci. USA, 91:10747-10751 [1994]; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767; WO 2009/152336, and U.S. Pat. No. 6,537,746. all of which are incorporated herein by reference).

[0204] In some embodiments, the enzyme clones obtained following mutagenesis treatment are screened by subjecting the enzyme preparations to a defined temperature (or other assay conditions) and measuring the amount of enzyme activity remaining after heat treatments or other suitable assay conditions. Clones containing a polynucleotide encoding a PAL polypeptide are then isolated from the gene, sequenced to identify the nucleotide sequence changes (if any), and used to express the enzyme

in a host cell. Measuring enzyme activity from the expression libraries can be performed using any suitable method known in the art (e.g., standard biochemistry techniques, such as HPLC analysis).

[0205] For engineered polypeptides of known sequence, the polynucleotides encoding the enzyme can be prepared by standard solid-phase methods, according to known synthetic methods. In some embodiments, fragments of up to about 100 bases can be individually synthesized, then joined (e.g., by enzymatic or chemical litigation methods, or polymerase mediated methods) to form any desired continuous sequence. For example, polynucleotides and oligonucleotides disclosed herein can be prepared by chemical synthesis using the classical phosphoramidite method (See e.g., Beaucage et al., Tet. Lett., 22:1859-69 [1981]; and Matthes et al., EMBO J., 3:801-05 [1984]), as it is typically practiced in automated synthetic methods. According to the phosphoramidite method, oligonucleotides are synthesized (e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors).

[0206] Accordingly, in some embodiments, a method for preparing the engineered PAL polypeptide can comprise: (a) synthesizing a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from the amino acid sequence of any variant as described herein, and (b) expressing the PAL polypeptide encoded by the polynucleotide. In some embodiments of the method, the amino acid sequence encoded by the polynucleotide can optionally have one or several (e.g., up to 3, 4, 5, or up to 10) amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-10, 1-15, 1-20, 1-21, 1-22, 1-23, 1-24, 1-25, 1-30, 1-35, 1-40, 1-45, or 1-50 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 30, 35, 40, 45, or 50 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the amino acid sequence has optionally 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 21, 22, 23, 24, or 25 amino acid residue deletions, insertions and/or substitutions. In some embodiments, the substitutions are conservative or non-conservative substitutions.

[0207] The expressed engineered PAL polypeptide can be evaluated for any desired improved property or combination of properties (e.g., activity, selectivity, stability, acid tolerance, protease sensitivity, etc.) using any suitable assay known in the art, including but not limited to the assays and conditions described herein.

[0208] In some embodiments, any of the engineered PAL polypeptides expressed in a host cell are recovered from the cells and/or the culture medium using any one or more of the well-known techniques for protein purification, including, among others, lysozyme treatment, sonication, filtration, salting-out, ultra-centrifugation, and chromatography.

[0209] Chromatographic techniques for isolation of the PAL polypeptides include, among others, reverse phase chromatography, high-performance liquid chromatography, ion-exchange chromatography, hydrophobic-interaction chromatography, size-exclusion chromatography, gel

electrophoresis, and affinity chromatography. Conditions for purifying a particular enzyme depends, in part, on factors such as net charge, hydrophobicity, hydrophilicity, molecular weight, molecular shape, etc., and will be apparent to those having skill in the art. In some embodiments, affinity techniques may be used to isolate the improved PAL enzymes. For affinity chromatography purification, any antibody that specifically binds a PAL polypeptide of interest may find use. For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, etc., are immunized by injection with a PAL polypeptide, or a fragment thereof. In some embodiments, the PAL polypeptide or fragment is attached to a suitable carrier, such as BSA, by means of a side chain functional group or linkers attached to a side chain functional group.

[0210] In some embodiments, the engineered PAL polypeptide is produced in a host cell by a method comprising culturing a host cell (e.g., an *E. coli* strain) comprising a polynucleotide sequence encoding an engineered PAL polypeptide as described herein under conditions conducive to the production of the engineered PAL polypeptide and recovering the engineered PAL polypeptide from the cells and/or culture medium. In some embodiments, the host cell produces more than one engineered PAL polypeptide.

[0211] In some embodiments, the present invention provides a method of producing an engineered PAL polypeptide comprising culturing a recombinant bacterial cell comprising a polynucleotide sequence encoding an engineered PAL polypeptide having at least 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to reference sequences SEQ ID NO:4, and one or more amino acid residue differences as compared to SEQ ID NO:4 selected from X39; X91; X158; X180; X195; X243; X245; X256; X257; X270; X290; X307; X308; X326; X349; X364; X394; X399; X400; X404; X407; X443; X453; X459; X460; X463; X474; X522; X524; and X528, or combinations thereof, when optimally aligned with the amino acid sequence of SEQ ID NO:4 under suitable culture conditions to allow the production of the engineered PAL polypeptide and optionally recovering the engineered PAL polypeptide from the culture and/or cultured bacterial cells. In some embodiments, the host cell produces more than one engineered PAL polypeptide.

[0212] In some embodiments, once the engineered PAL polypeptides are recovered from the recombinant host cells and/or culture medium, they are further purified by any suitable method(s) known in the art. In some additional embodiments, the purified TAL polypeptides are combined with other ingredients and compounds to provide compositions and formulations comprising the engineered PAL polypeptide as appropriate for different applications and uses (e.g., pharmaceutical compositions).

Compositions:

[0213] The present invention provides engineered PAL polypeptides suitable for use in numerous compositions. These compositions find use in many fields, including but not limited to pharmaceuticals, dietary/nutritional supplements, food, feed, and fine chemical production. For

example, in some embodiments, the present invention provides food and/or feeds comprising at least one engineered PAL variant and/or at least one polynucleotide sequence encoding at least one PAL variant. In some embodiments, the present invention provides beverages comprising at least one engineered PAL variant.

[0214] In some embodiments, the engineered PAL variant in food, feed, and/or nutritional/dietary supplement is glycosylated. Furthermore, the engineered PAL variants find use in any suitable edible enzyme delivery matrix. In some embodiments, the engineered PAL variants are present in an edible enzyme delivery matrix designed for rapid dispersal of the PAL variant within the digestive tract of an animal upon ingestion of the variant.

[0215] The present invention also provides engineered PAL polypeptides suitable for use in production of fine chemicals and other industrially important compounds (See e.g., US Pat. Appln. Nos. 2013/0340119, 2013/0005012, and 2005/0260724, and WO 2012/122333).

Pharmaceutical and Other Compositions

[0216] The present invention provides engineered PAL polypeptides suitable for use in pharmaceutical and other compositions, such as dietary/nutritional supplements.

[0217] Depending on the mode of administration, a these compositions comprising a therapeutically effective amount of an engineered PAL according to the invention are in the form of a solid, semi-solid, or liquid. In some embodiments, the compositions include other pharmaceutically acceptable components such as diluents, buffers, excipients, salts, emulsifiers, preservatives, stabilizers, fillers, and other ingredients. Details on techniques for formulation and administration are well known in the art and described in the literature.

[0218] In some embodiments, the engineered PAL polypeptides are formulated for use in oral pharmaceutical compositions. Any suitable format for use in delivering the engineered PAL polypeptides find use in the present invention, including but not limited to pills, tablets, gel tabs, capsules, lozenges, dragees, powders, soft gels, sol-gels, gels, emulsions, implants, patches, sprays, ointments, liniments, creams, pastes, jellies, paints, aerosols, chewing gums, demulcents, sticks, suspensions (including but not limited to oil-based suspensions, oil-in water emulsions, etc.), slurries, syrups, controlled release formulations, suppositories, etc. In some embodiments, the engineered PAL polypeptides are provided in a format suitable for injection (i.e., in an injectable formulation). In some embodiments, the engineered PAL polypeptides are provided in biocompatible matrices such as sol-gels, including silica-based (e.g., oxysilane) sol-gels. In some embodiments, the engineered PAL polypeptides are encapsulated. In some alternative embodiments, the engineered PAL polypeptides are encapsulated in nanostructures (e.g., nanotubes, nanotubules, nanocapsules, or microcapsules, microspheres, liposomes, etc.). Indeed, it is not intended that the present invention be limited to any particular delivery formulation and/or means of delivery. It is intended that the engineered PAL

polypeptides be administered by any suitable means known in the art, including but not limited to parenteral, oral, topical, transdermal, intranasal, intraocular, intrathecal, via implants, etc.

[0219] In some embodiments, the engineered PAL polypeptides are chemically modified by glycosylation, pegylation (i.e., modified with polyethylene glycol [PEG] or activated PEG, etc.) or other compounds (See e.g., Ikeda, Amino Acids 29:283-287 [2005]; US Pat. Nos. 7,531,341, 7,534,595, 7,560,263, and 7,53,653; US Pat. Appln. Publ. Nos. 2013/0039898, 2012/0177722, etc.). Indeed, it is not intended that the present invention be limited to any particular delivery method and/or mechanism.

[0220] In some additional embodiments, the engineered PAL polypeptides are provided in formulations comprising matrix-stabilized enzyme crystals. In some embodiments, the formulation comprises a cross-linked crystalline engineered PAL enzyme and a polymer with a reactive moiety that adheres to the enzyme crystals. The present invention also provides engineered PAL polypeptides in polymers.

[0221] In some embodiments, compositions comprising the engineered PAL polypeptides of the present invention include one or more commonly used carrier compounds, including but not limited to sugars (e.g., lactose, sucrose, mannitol, and/or sorbitol), starches (e.g., corn, wheat, rice, potato, or other plant starch), cellulose (e.g., methyl cellulose, hydroxypropylmethyl cellulose, sodium carboxy-methylcellulose), gums (e.g., arabic, tragacanth, guar, etc.), and/or proteins (e.g., gelatin, collagen, etc.). Additional components in oral formulations may include coloring and or sweetening agents (e.g., glucose, sucrose, and mannitol) and lubricating agents (e.g., magnesium stearate), as well as enteric coatings (e.g., methacrylate polymers, hydroxyl propyl methyl cellulose phthalate, and/or any other suitable enteric coating known in the art). In some embodiments, disintegrating or solubilizing agents are included (e.g., cross-linked polyvinyl pyrrolidone, agar, alginic acid or salts thereof, such as sodium alginate). In some embodiments, the engineered PAL polypeptide are combined with various additional components, including but not limited to preservatives, suspending agents, thickening agents, wetting agents, alcohols, fatty acids, and/or emulsifiers, particularly in liquid formulations.

[0222] In some embodiments, the engineered PAL polypeptide are be combined with various additional components, including but not limited to preservatives, suspending agents, thickening agents, wetting agents, alcohols, fatty acids, and/or emulsifiers, particularly in liquid formulations. In some embodiments, the engineered PAL polypeptides are administered to subjects in combination with other compounds used in the treatment of PKU, including but not limited to KUVAN® tetrahydrobiopterin (BioMarin Pharmaceutical, Inc., Novato, CA), antacids (e.g., omeprazole, esomeprazole and other prazoles), as well as any other suitable compounds.

[0223] In some embodiments, the present invention provides engineered PAL polypeptides suitable for use in decreasing the concentration of phenylalanine in fluids such as blood, cerebrospinal fluid, etc. The dosage of engineered PAL polypeptide(s) administered to an animal depend upon the

condition or disease, the general condition of the animal, and other factors known to those in the art. In some embodiments, the compositions are intended for single or multiple administration to an animal. In some embodiments, it is contemplated that the concentration of engineered PAL polypeptide(s) in the composition(s) administered to an animal (e.g., a human with PKU) is sufficient to effectively treat, ameliorate and/or prevent disease (e.g., PKU and/or PKU-related conditions, diseases and/or symptoms), In some embodiments, the engineered PAL polypeptides are administered in combination with other pharmaceutical and/or dietary compositions.

Industrial Compositions

[0224] It is contemplated that the engineered PAL polypeptides of the present invention will find use in industrial compositions. In some embodiments, the engineered PAL polypeptides are formulated for use in the food and/or feed industries. In some embodiments, the engineered PAL polypeptides are formulated in granulated or pelleted products which are mixed with animal feed components such as additional enzymes (for example, cellulases, laccases, and amylases). In some alternative embodiments, the engineered PAL polypeptides are used in liquid animal feed compositions (e.g., aqueous or oil based slurries). Thus, in some embodiments, the engineered PAL variants of the present invention are sufficiently thermotolerant and thermostable to withstand the treatment used to produce pellets and other processed feed/foods.

[0225] The engineered PAL variants of the present invention also find use in the production of phenylalanine and/or phenylalanine derivatives.

[0226] The foregoing and other aspects of the invention may be better understood in connection with the following non-limiting examples. The examples are provided for illustrative purposes only and are not intended to limit the scope of the present invention in any way.

EXPERIMENTAL

[0227] The following examples, including experiments and results achieved, are provided for illustrative purposes only and are not to be construed as limiting the present invention.

[0228] In the experimental disclosure below, the following abbreviations apply: ppm (parts per million); M (molar); mM (millimolar), uM and μM (micromolar); nM (nanomolar); mol (moles); gm and g (gram); mg (milligrams); ug and μg (micrograms); L and l (liter); ml and mL (milliliter); cm (centimeters); mm (millimeters); um and μm (micrometers); sec. (seconds); min(s) (minute(s)); h(s) and hr(s) (hour(s)); U (units); MW (molecular weight); rpm (rotations per minute); psi and PSI (pounds per square inch); °C (degrees Centigrade); RT and rt (room temperature); CDS (coding sequence); DNA (deoxyribonucleic acid); RNA (ribonucleic acid); *E. coli* W3110 (commonly used laboratory *E. coli* strain, available from the Coli Genetic Stock Center [CGSC], New Haven, CT); HTP (high throughput); HPLC (high pressure liquid chromatography); CFSE (carboxyfluorescein succinimidyl ester); IPTG (isopropyl β-D-1-thiogalactopyranoside); PES (polyethersulfone); PHE and

phe (phenylalanine); BSA (bovine serum albumin); PBMC (peripheral blood mononuclear cells); PKU (phenylketonuria); MHC (major histocompatibility complex); HLA (human leukocyte antigen); HLA-DR (an MHC Class II cell surface receptor encoded by the HLA complex on chromosome #6); FIOPC (fold improvements over positive control); LB (Luria broth); Athens Research (Athens Research Technology, Athens, GA); ProSpec (ProSpec Tany Technogene, East Brunswick, NJ); Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO); Ram Scientific (Ram Scientific, Inc., Yonkers, NY); Pall Corp. (Pall, Corp., Pt. Washington, NY); Millipore (Millipore, Corp., Billerica MA); Difco (Difco Laboratories, BD Diagnostic Systems, Detroit, MI); Molecular Devices (Molecular Devices, LLC, Sunnyvale, CA); Kuhner (Adolf Kuhner, AG, Basel, Switzerland); Cambridge Isotope Laboratories, (Cambridge Isotope Laboratories, Inc., Tewksbury, MA); Applied Biosystems (Applied Biosystems, part of Life Technologies, Corp., Grand Island, NY), Agilent (Agilent Technologies, Inc., Santa Clara, CA); Thermo Scientific (part of Thermo Fisher Scientific, Waltham, MA); Corning (Corning, Inc., Palo Alto, CA); Constant Systems (Constant Systems Ltd., Daventry, United Kingdom); Megazyme (Megazyme International, Wicklow, Ireland); Enzo (Enzo Life Sciences, Inc., Farmingdale, NY); GE Healthcare (GE Healthcare Bio-Sciences, Piscataway, NJ); Harlan (Harlan Laboratories, Indianapolis, IN); AB Sciex (AB Sciex, Framingham, MA); and Bio-Rad (Bio-Rad Laboratories, Hercules, CA).

[0229] The following polynucleotide and polypeptide sequences find use in the present invention. In some cases (as shown below), the polynucleotide sequence is followed by the encoded polypeptide.

Polynucleotide Sequence of pET16b-AvPAL Expression Vector (SEQ ID NO:1):

TCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAGTTTATCACAGTTAAATT
GCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAATGCGCTCATCGTCATCCTCGGCA
CCGTCACCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCCTCTTGC
GGGATATCCGGATATAGTTCCTCCTTTCAGCAAAAAACCCCTCAAGACCCGTTTAGAGGC
CCCAAGGGGTTATGCTAGTTATTGCTCAGCGGTGGCAGCAGCCAACTCAGCTTCCTTTCG
GGCTTTGTTAGCAGCCGGATCCTTAATGCAGACACGGCAGAATGTCCTGAACGGCCTGA
ACAATAACACCACCGGCTGCAATATCTGCACTAATACGTGCAATATGTTCATCCAGACCC
TGTTCATTATCATTCCAAATATACGGACGATCTGAGGTCGGTTTCTGACCAACAACATGA
CGAACTGCGCTATACAGACGTTCGGTTGCCGGTGACAGACAGGCACGTGCATCATAATG
ACCGGTTTTTTTGTAGGTACGCAGATCAACTGCCTGAACACCAAACATCAGGGCAATGGC
AACATAATTCTGAAAAATATCAACGCTACGACGTGCCAGGGTTGCGCTGGTATAACCCTG
GCTGTTAATATTCTGGTTAAACTGTTCGGCATGGGTCGGAAAACGATCTGCAATACTATT
ACCATAAAAGGTCAGCAGCGGCATAATGCTATTACCGCAAATCTGCAGACCTTTCAGAC
CCATATTAACTTTACGTTCACGATTACCCAGCAGACTCGGAGGCAGACCATTGCTAAATT
CCGGTGATGCCAGCAGTGCAATCTGAACATCCAGATGTTTTGCCAGCAGACCGATATAAT
AGCGCAGATGATCCATACCCATACCAACATACTGACCCAGAAAATTACCACCATGATAG
CTTGCCTGATTATCAACATCAATCAGCGGGTTATCGGTAACGCTGTTAATCTCAATTTCG
ATTTGTTTGGCAATCTGGCTAATACCATCAACAATCGGACCCAGATACTGCGGCAGACAA
CGCAGGCTATAACGATCCTGGATCAGTTCATGATCACGATAATCATGTTTACCATCCAGT
TCATCACGAACCAGCTGGCTATTGGCCAGCAGGCTAATCATCTGATCTGCTGCCCACAGC

TGACCCGGATGCGGTTTGCTGTTATGGATAAACGGATGAAAGCTCTGATTTGTACCATTC
AGTGCCTGAATATCCAGTGCATGAACACCCATTGCAATTGCGGTCAGAATCTGGGTATCA
TAAACACAATTTGCTGCAATACCGGTCATAACGCTGGTGCCATTCATCATTGCCAGACCT
TCTTTCGGCAGCAGGGTCAGCGGACTCAGATTCAGCTGACGCAGTGCGGTCGGTGCGTCC
ATTTCTTTGCCATTAAAATCAACTTTAAAGCTCGGGTCCAGGCCAATCAGGCTACCGGTA
ATATAGCTCAGCGGAACCAGATCACCGCTGGCACCAATGCTACCAAATTCATAAACATA
CGGGGTAACACCGGCATTCAGAAAGATTTCCATGCGTTTAATCAGTTCCAGACGAATACC
GCTTGCACCACGCATGTGGCTATTTGCACGCAGCAGCATTGCTGCACGAACATCTGCCAG
CGGCAGTTTATTACCTGCACCGGTTTTCAGAAACCAAACCAGATTGGTCTGCAGTTCGCT
TGCCTGTTCACGGCTAATTGCAACATTTGCCATACCACCAAAACCGCTGGTAACACCATA
AATCGGTTCACCGCTTTCAACTGCATTATTGATATAATCACAGCTGGCCTGAATACCCTG
CAGAATATCGGTATTATTGGTCAGGCTAACCAGGGTGCCATTACGGGCAACACGTGCAA
CATCATTGATGGTCAGTTTCTGATTACCAATAATCACATTTGCGCTGCTATTGCCGGTAAA
GCTAAACTGCTGGCTGCTGGTTTTGCTCTGTGCCTGGCTCAGGGTTTTCATATGACGACCT
TCGATATGGCCGCTGCTGTGATGATGATGATGATGATGATGATGGCCCATGGTATAT
CTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCC
CCTATAGTGAGTCGTATTAATTTCGCGGGATCGAGATCTCGATCCTCTACGCCGGACGCA
TCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCA
CCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTA
TGGTGGCAGGCCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCC
TTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGT
CGCATAAGGGAGAGCGTCGAGATCCCGGACACCATCGAATGGCGCAAAACCTTTCGCGG
TATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGAAACCAGTAA
CGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGA
ACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGGA
GCTGAATTACATTCCCAACCGCGTGGCACAACAACTGGCGGGCAAACAGTCGTTGCTGA
TTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTA
AATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGC
GTCGAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGAT
CATTAACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGT
TCCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCAT
GAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGC
GCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAA
ATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCA
TGTCCGGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGC
TGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTG
CGCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTAT
ATCCCGCCGTTAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGAC
CGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCA
CTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTT
GGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAG
CGCAACGCAATTAATGTAAGTTAGCTCACTCATTAGGCACCGGGATCTCGACCGATGCCC
TTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATGACTATCGTCGCC
GCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGG
GTCATTTTCGGCGAGGACCGCTTTCGCTGGAGCGCGACGATGATCGGCCTGTCGCTTGCG
GTATTCGGAATCTTGCACGCCCTCGCTCAAGCCTTCGTCACTGGTCCCGCCACCAAACGT
TTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCTGGGCTACGTCTT
GCTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCCATTATGATTCTTCTCGCTTCCGG

CGGCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATC
AGGGACAGCTTCAAGGATCGCTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGC
TGATCGTCACGGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTG
TAGGCGCCGCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGG
CCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGATTCACCACTCCAAGAA
TTGGAGCCAATCAATTCTTGCGGAGAACTGTGAATGCGCAAACCAACCCTTGGCAGAAC
ATATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATCTCGGGCAGCGTTGGG
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GGTTGCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAACGTGAAGCGACTG
CTGCTGCAAAACGTCTGCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTC
GTAAAGTCTGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCATCGC
AGGATGCTGCTGGCTACCCTGTGGAACACCTACATCTGTATTAACGAAGCGCTGGCATTG
ACCCTGAGTGATTTTTCTCTGGTCCCGCCGCATCCATACCGCCAGTTGTTTACCCTCACAA
CGTTCCAGTAACCGGGCATGTTCATCATCAGTAACCCGTATCGTGAGCATCCTCTCTCGTT
TCATCGGTATCATTACCCCCATGAACAGAAATCCCCCTTACACGGAGGCATCAGTGACCA
AACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAACGCTT
CTGGAGAAACTCAACGAGCTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTCA
CGACCACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAA
CCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGA
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ACCCAGTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCAG
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GGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCA
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CCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAG
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CCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATC
GCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTA
CAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCT
GCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAC
AAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAA
AAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAA
ACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTT
TAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACA
GTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCAT
AGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCC
CAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAA
ACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATC
CAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGC
AACGTTGTTGCCATTGCTGCAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCAT
TCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAG
CGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCAC
TCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC
TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTG

CTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCT
CATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATC
CAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGC
GTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGA
CACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGG
TTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGT
TCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGAC
ATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAAT(SEQ ID NO:1)

Polynucleotide Sequence of the AvPAL ORF (SEQ ID NO:2):

ATGAAAACCCTGAGCCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGG
CAATAGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGCAC
GTGTTGCCCGTAATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTA
TTCAGGCCAGCTGTGATTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTG
TTACCAGCGGTTTTGGTGGTATGGCAAATGTTGCAATTAGCCGTGAACAGGCAAGCGAA
CTGCAGACCAATCTGGTTTGGTTTCTGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCA
GATGTTCGTGCAGCAATGCTGCTGCGTGCAAATAGCCACATGCGTGGTGCAAGCGGTATT
CGTCTGGAACTGATTAAACGCATGGAAATCTTTCTGAATGCCGGTGTTACCCCGTATGTT
TATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCCGCTGAGCTATATTACCGGT
AGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCAAAGAAATGGACGC
ACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAAAGAAGGTCT
GGCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTATGATAC
CCAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGGCACTGAATGG
TACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTG
GGCAGCAGATCAGATGATTAGCCTGCTGGCCAATAGCCAGCTGGTTCGTGATGAACTGG
ATGGTAAACATGATTATCGTGATCATGAACTGATCCAGGATCGTTATAGCCTGCGTTGTC
TGCCGCAGTATCTGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAA
TTGAGATTAACAGCGTTACCGATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATC
ATGGTGGTAATTTTCTGGGTCAGTATGTTGGTATGGGTATGGATCATCTGCGCTATTATAT
CGGTCTGCTGGCAAAACATCTGGATGTTCAGATTGCACTGCTGGCATCACCGGAATTTAG
CAATGGTCTGCCTCCGAGTCTGCTGGGTAATCGTGAACGTAAAGTTAATATGGGTCTGAA
AGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCTGACCTTTTATGGTAATAGTAT
TGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATATTAACAGCCAGGGTTA
TACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTTTTCAGAATTATGTTGCCATTGC
CCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCATTATGA
TGCACGTGCCTGTCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTGT
TGGTCAGAAACCGACCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGG
ATGAACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCG
TTCAGGACATTCTGCCGTGTCTGCAT (SEQ ID NO:2)

Polynucleotide Sequence of WT AvPAL (SEQ ID NO:3):

ATGAAAACCCTGAGCCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGG
CAATAGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGCAC

GTGTTGCCCGTAATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTA
TTCAGGCCAGCTGTGATTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTG
TTACCAGCGGTTTTGGTGGTATGGCAAATGTTGCAATTAGCCGTGAACAGGCAAGCGAA
CTGCAGACCAATCTGGTTTGGTTTCTGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCA
GATGTTCGTGCAGCAATGCTGCTGCGTGCAAATAGCCACATGCGTGGTGCAAGCGGTATT
CGTCTGGAACTGATTAAACGCATGGAAATCTTTCTGAATGCCGGTGTTACCCCGTATGTT
TATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCCGCTGAGCTATATTACCGGT
AGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCAAAGAAATGGACGC
ACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAAAGAAGGTCT
GGCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTATGATAC
CCAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGGCACTGAATGG
TACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTG
GGCAGCAGATCAGATGATTAGCCTGCTGGCCAATAGCCAGCTGGTTCGTGATGAACTGG
ATGGTAAACATGATTATCGTGATCATGAACTGATCCAGGATCGTTATAGCCTGCGTTGTC
TGCCGCAGTATCTGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAA
TTGAGATTAACAGCGTTACCGATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATC
ATGGTGGTAATTTTCTGGGTCAGTATGTTGGTATGGGTATGGATCATCTGCGCTATTATAT
CGGTCTGCTGGCAAAACATCTGGATGTTCAGATTGCACTGCTGGCATCACCGGAATTTAG
CAATGGTCTGCCTCCGAGTCTGCTGGGTAATCGTGAACGTAAAGTTAATATGGGTCTGAA
AGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCTGACCTTTTATGGTAATAGTAT
TGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATATTAACAGCCAGGGTTA
TACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTTTTCAGAATTATGTTGCCATTGC
CCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCATTATGA
TGCACGTGCCTGTCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTGT
TGGTCAGAAACCGACCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGG
ATGAACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCG
TTCAGGACATTCTGCCGTGTCTGCAT (SEQ ID NO:3)

Polypeptide Sequence of WT AvPAL (SEQ ID NO:4):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVARVARNGTLVSLTNNTDILQGIQAS
CDYINNAVESGEPIYGVTSGFGGMANVAISREQASELQTNLVWFLKTGAGNKLPLADVRAA
MLLRANSHMRGASGIRLELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDPSF
KVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAMGV
HALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLANSQLVRDELDGKHDYRDHELIQ
DRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDH
LRYYIGLLAKHLDVQIALLASPEFSNGLPPSLLGNRERKVNMGLKGLQICGNSIMPLLTFYGN
SIADRFPTHAEQFNQNINSQGYTSATLARRSVDIFQNYVAIALMFGVQAVDLRTYKKTGHYD
ARACLSPATERLYSAVRHVVGQKPTSDRPYIWNDNEQGLDEHIARISADIAAGGVIVQAVQDI
LPCLH (SEQ ID NO:4)

Polynucleotide Sequence of AvPAL Variant No. 30 (SEQ ID NO:5):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGG
CAATAGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGTACG
TGTTGCCCGTAATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTAT
TCAGGCCAGCTGTGATTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTGT
TACCAGCGGTTTTGGTGGTATGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACT

GCAGACCAATCTGGTTTGGTTTCTGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCAG
ATGTTCGTGCAGCAATGCTGCTGCGTGCAAATAGCCACATGCGTGGTGCAAGCGGTATTC
GTCTGGAACTGATTAAACGCATGGAAATCTTTCTGAATGCCGGTGTTACCCCGTATGTTT
ATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCCGCTGAGCTATATTACCGGTA
GCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCAAAGAAATGGACGCAC
CGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAAAGAAGGTCTG
GCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTATGATACC
CAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGGGACTGAATGGT
ACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTGG
GCAGCAGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGGA
TGGTAAACATGATTATCGTGATCATGAACTGATCCAGGATCGTTATAGCCTGCGTTGTCT
GCCGCAGTATCTGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAAT
TGAGATTAACAGCGTTACCGATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATCA
TGGTGGTAATTTTCTGGGTCAGTATGTTGGTATGGGTATGGATCATCTGCGCTATTATATC
GGTCTGCTGGCAAAACATCTGGATGTTCAGATTGCACTGCTGGTATCACCGGAATTTAAC
AATGGTCTGCCTGCGAGTCTGGTGGGTAATCGTGAACGTAAAGTTAATATGGGTCTGAAA
GGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCTGACCTTTTATGGTAATAGTATT
GCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATATTAACAGCCAGGGTTAT
ACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTTTTCAGAATTATGTTGCCATTGCC
CTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCATTATGAT
GCACGTGCCTGTCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTGTT
GGTCAGTATCCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGGAT
GAACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCGTT
CAGGACATTCTGCCGTGTCTGCAT (SEQ ID NO:5)

Polypeptide Sequence of AvPAL Variant No. 30 (SEQ ID NO:6):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVVRVARNGTLVSLTNNTDILQGIQAS
CDYINNAVESGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADVRAA
MLLRANSHMRGASGIRLELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDPSF
KVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAMGV
HALDIQGLNGTNQSFHPFIHNSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYRDHELIQ
DRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDH
LRYYIGLLAKHLDVQIALLVSPEFNNGLPASLVGNRERKVNMGLKGLQICGNSIMPLLTFYG
NSIADRFPTHAEQFNQNINSQGYTSATLARRSVDIFQNYVAIALMFGVQAVDLRTYKKTGHY
DARACLSPATERLYSAVRHVVGQYPSSDRPYIWNDNEQGLDEHIARISADIAAGGVIVQAVQ
DILPCLH (SEQ ID NO:6)

Polynucleotide Sequence of AvPAL Variant No. 22 (SEQ ID NO:7):

ATGAAAACCCTGAGCCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGG
CAATAGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGCAC
GTGTTGCCCGTAATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTA
TTCAGGCCAGCTGTGATTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTG
TTACCAGCGGTTTTGGTGGTATGGCAAATGTTGCAATTAGCCGTGAACAGGCAAGCGAA

CTGCAGACCAATCTGGTTTGGTTTCTGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCA
GATGTTCGTGCAGCAATGCTGCTGCGTGCAAATAGCCACATGCGTGGTGCAAGCGGTATT
CGTCTGGAACTGATTAAACGCATGGAAATCTTTCTGAATGCCGGTGTTACCCCGTATGTT
TATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCCGCTGAGCTATATTACCGGT
AGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCAAAGAAATGGACGC
ACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAAAGAAGGTCT
GGCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTATGATAC
CCAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGGCACTGAATGG
TACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTG
GGCAGCAGATCAGATGATTAGCCTGCTGGCCAATAGCCAGCTGGTTCGTGATGAACTGG
ATGGTAAACATGATTATCGTGATGGTGAACTGATCCAGGATCGTTATAGCCTGCGTTGTC
TGCCGCAGTATCTGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAA
TTGAGATTAACAGCGTTACCGATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATC
ATGGTGGTAATTTTCTGGGTCAGTATGTTGGTATGGGTATGGATCATCTGCGCTATTATAT
CGGTCTGCTGGCAAAACATCTGGATGTTCAGATTGCACTGCTGGCATCACCGGAATTTAG
CAATGGTCTGCCTCCGAGTCTGCTGGGTAATCGTGAACGTAAAGTTAATATGGGTCTGAA
AGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCTGACCTTTTATGGTAATAGTAT
TGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATATTAACAGCCAGGGTTA
TACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTTTTCAGAATTATGTTGCCATTGC
CCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCATTATGA
TGCACGTGCCTGTCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTGT
TGGTCAGAAACCGACCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGG
ATGAACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCG
TTCAGGACATTCTGCCGTGTCTGCAT (SEQ ID NO:7)

Polypeptide Sequence of AvPAL Variant No. 22 (SEQ ID NO:8):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVARVARNGTLVSLTNNTDILQGIQAS
CDYINNAVESGEPIYGVTSGFGGMANVAISREQASELQTNLVWFLKTGAGNKLPLADVRAA
MLLRANSHMRGASGIRLELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDPSF
KVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAMGV
HALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLANSQLVRDELDGKHDYRDGELIQ
DRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDH
LRYYIGLLAKHLDVQIALLASPEFSNGLPPSLLGNRERKVNMGLKGLQICGNSIMPLLTFYGN
SIADRFPTHAEQFNQNINSQGYTSATLARRSVDIFQNYVAIALMFGVQAVDLRTYKKTGHYD
ARACLSPATERLYSAVRHVVGQKPTSDRPYIWNDNEQGLDEHIARISADIAAGGVIVQAVQDI
LPCLH (SEQ ID NO:8)

Polynucleotide Sequence of AvPAL Variant No. 36 (SEQ ID NO:9):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGG
CAATAGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGTACG
TGTTGCCCGTAATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTAT
TCAGGCCAGCTGTGATTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTGT
TACCAGCGGTTTTGGTGGTATGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACT
GCAGACCAATCTGGTTTGGTTTCTGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCAG
ATGTTCGTGCAGCAATGCTGCTGCGTGCAAATAGCCACATGCGTGGTGCAAGCGGTATTC
GTCTGGAACTGATTAAACGCATGGAAATCTTTCTGAATGCCGGTGTTACCCCGTATGTTT
ATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCCGCTGAGCTATATTACCGGTA

GCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCAAAGAAATGGACGCAC
CGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAAAGAAGGTCTG
GCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTATGATACC
CAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGGCACTGAATGGT
ACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTGG
GCAGCAGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGGA
TGGTAAACATGATTATCGTGATGGTGAACTGATCCAGGATCGTTATAGCCTGCGTTGTCT
GCCGCAGTATCTGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAAT
TGAGATTAACAGCGTTACCGATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATCA
TGGTGGTAATTTTCTGGGTCAGTATGTTGGTATGGGTATGGATCATCTGCGCTATTATATC
GGTCTGCTGGCAAAACATCTGGATGTTCAGATTGCACTGCTGGCATCACCGGAATTTAGC
AATGGTCTGCCTCCGAGTCTGGTGGGTAATCGTGAACGTAAAGTTAATATGGGTCTGAAA
GGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCTGACCTTTTATGGTAATAGTATT
GCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATATTAACAGCCAGGGTTAT
ACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTTTTCAGAATTATGTTGCCATTGCC
CTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCATTATGAT
GCACGTGCCTGTCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTGTT
GGTCAGAAACCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGGA
TGAACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCGT
TCAGGACATTCTGCCGTGTCTGCAT (SEQ ID NO:9)

Polypeptide Sequence of AvPAL Variant No. 36 (SEQ ID NO:10):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVVRVARNGTLVSLTNNTDILQGIQAS
CDYINNAVESGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADVRAA
MLLRANSHMRGASGIRLELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDPSF
KVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAMGV
HALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYRDGELIQ
DRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDH
LRYYIGLLAKHLDVQIALLASPEFSNGLPPSLVGNRERKVNMGLKGLQICGNSIMPLLTFYGN
SIADRFPTHAEQFNQNINSQGYTSATLARRSVDIFQNYVAIALMFGVQAVDLRTYKKTGHYD
ARACLSPATERLYSAVRHVVGQKPSSDRPYIWNDNEQGLDEHIARISADIAAGGVIVQAVQDI
LPCLH (SEQ ID NO:10)

Polynucleotide Sequence of AvPAL Variant No. 42 (SEQ ID NO:11):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGGC
AATAGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGTACGT
GTTGCCCGTAATGGCACCCTGGTTAGCCTGACCAATAATAAAGATATTCTGCAGCGTATTCAGGC
CAGCTGTGATTATATCAATAATGCAGTTGAAAAAGGTGAACCGATTTATGGTGTTACCAGCGGTT
TTGGTGGTATGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACTGCAGACCAATCTGGT
TTGGTTTCTGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCAGATGTTCGTGCAGCAATGCTGC
TGCGTGCAAATAGCCACATGCGTGGTGCAAGCGGTATTCGTCTGGAACTGATTAAACGCATGGA
AATCTTTCTGAATGCCGGTGTTACCCCGTATGTTTATGAATTTGGTAGCATTGGTGCCAGCGGTG
ATCTGGTTCCGCTGAGCTATATTACCGGTAGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGAT
TTTAATGGCAAAGAAATGGACGCACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCC
TGCTGCCGAAAGAAGGTCTGGCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAA

TTGTGTTTATGATACCCAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGG
CACTGAATGGTACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAG
CTGTGGGCAGCAGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGG
ATGGTAAACATGATTATATGGATGGTGAACTGATCCAGGATCGTTATAGCCTGCGTTGTCTGCCG
CAGTATCTGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAATTGAGATTAA
CAGCGTTACCGATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATCATGGTGGTAATTTTC
TGGGTCAGTATGTTGGTATGGGTATGGATCATCTGCGCTATTATATCGGTCTGCTGGCAAAACAT
CTGGATGTTCAGATTGCACTGCTGGCATCACCGGAATTTAGCAATGGTCTGCCTCCGAGTCTGGT
GGGTAATCGTGAACGTAAAGTTAATATGGGTCTGAAAGGTCTGCAGATTTGCGGTAATAGCATT
ATGCCGCTGCTGACCTTTTATGGTAATAGTATTGCAGATCGTTTTCCGACCCATGCCGAACAGTT
TAACCAGAATATTAACAGCCAGGGTTATACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTT
TTCAGAATTATGTTGCCATTGCCCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAA
AAAACCGGTCATTATGATGCACGTGCCCAGCTGTCACCGGCAACCGAACGTCTGTATAGCGCAG
TTCGTCATGTTGTTGGTAAAAAACCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAG
GGTCTGGATGAACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCA
GGCCGTTCAGGACATTCTGCCGCCGCTGCAT (SEQ ID NO:11)

Polypeptide Sequence of AvPAL Variant No. 42 (SEQ ID NO:12):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVVRVARNGTLVSLTNNKDILQRIQASCDYIN
NAVEKGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADVRAAMLLRANSHM
RGASGIRLELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDPSFKVDFNGKEMDAPTA
LRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAMGVHALDIQALNGTNQSFHPFIH
NSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYMDGELIQDRYSLRCLP
QYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDHLRYYIGLLAKHLDV
QIALLASPEFSNGLPPSLVGNRERKVNMGLKGLQICGNSIMPLLTFYGNSIADRFPTHAEQFNQNINSQ
GYTSATLARRSVDIFQNYVAIALMFGVQAVDLRTYKKTGHYDARAQLSPATERLYSAVRHVVGKKP
SSDRPYIWNDNEQGLDEHIARISADIAAGGVIVQAVQDILPPLH (SEQ ID NO:12)

Polynucleotide Sequence of AvPAL Variant No. 43 (SEQ ID NO:13):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGGCAATA
GCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGTACGTGTTGCCCGT
AATGGCACCCTGGTTAGCCTGACCAATAATAAAGATATTCTGCAGCGTATTCAGGCCAGCTGTG
ATTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTGTTACCAGCGGTTTTGGTGGT
ATGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACTGCAGACCAATCTGGTTTGGTTTCT
GAAAACCGGTGCAGGTAATAAACTGCCGCTGGCAGATGTTCGTGCAGCAATGCTGCTGCGTGCA
AATAGCCACATGCGTGGTGCAAGCGGTATTCGTCTGGAACTGATTAAACGCATGGAAATCTTTCT
GAATGCCGGTGTTACCCCGTATGTTTATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTC
CGCTGAGCTATATTACCGGTAGCCTGATTGGCC TGGACCCGAGCTTTAAAGTTGATTTTAATG
GCAAAGAAATGGACGCACCGACCGCACTGC
GTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAAAGAAGGTCTGGCAATGATGAATGGCAC
CAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTATGATACCCAGATTCTGACCGCAATTGCAA
TGGGTGTTCATGCACTGGATATTCAGGCACTGAATGGTACAAATCAGAGCTTTCATCCGTTTATC
CATAACAGCAAACCGCATCCGGGTCAGCTGTGGGCAGCAGATCAGATGATTAGCCTGCTGGCCG
GTAGCCAGCTGGTTCGTGATGAACTGGATGGTAAACATGATTATATGGATGGTGAACTGATCCA
GGATCGTTATAGCCTGCGTTGTCTGCCGCAGTATCTGGGTCCGATTGTTGATGGTATTAGCCAGA
TTGCCAAACAAATCGAAATTGAGATTAACAGCGTTACCGATAACCCGCTGATTGATGTTGATAAT
CAGGCAAGCTATCATGGTGGTAATTTTCTGGGTCAGTA
TGTTGGTATGGGTATGGATCATCTGCGCTATTATATCGGTCTGCTGGCAAAACATCTGGATGTTC
AGATTGCACTGCTGGCATCACCGGAATTTAGCAATGGTCTGCCTCCGAGTCTGGTGGGTAATCGT
GAACGTAAAGTTAATATGGGTCTGAAAGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCT

GACCTTTTATGGTAATAGTATTGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATA
TTAACAGCCAGGGTTATACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTTTTCAGAATTAT
GTTGCCATTGCCCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCA
TTATGATGCACGTGCCCAGCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTG
TTGGTAAAAAACCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGGATGA
ACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCGTTCAGGACA
TTCTGCCGAACCTGCAT (SEQ ID NO:13)

Polypeptide Sequence of AvPAL Variant No. 43 (SEQ ID NO:14):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVVRVARNGTLVSLTNNKDILQRIQASCDYIN
NAVESGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADVRAAMLLRANSHM
RGASGIRLELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDPSFKVDFNGKEMDAPTA
LRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAMGVHALDIQALNGTNQSFHPFIH
NSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYMDGELIQDRYSLRCLPQYLGPIVDGISQIAKQ
IEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDHLRYYIGLLAKHLDVQIALLASPEFSNGLPP
SLVGNRERKVNMGLKGLQICGNSIMPLLTFYGNSIADRFPTHAEQFNQNINSQGYTSATLARRSVDIF
QNYVAIALMFGVQAVDLRTYKKTGHYDARAQLSPATERLYSAVRHVVGKKPSSDRPYIWNDNEQG
LDEHIARISADIAAGGVIVQAVQDILPNLH (SEQ ID NO:14)

Polynucleotide Sequence of AvPAL Variant No. 1002 (SEQ ID NO:15):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGGCAATA
GCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGCGCGTGTTGCCCGT
AATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTATTCAGGCCAGCTGTGA
TTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTGTTACCAGCGGTTTTGGTGGTA
TGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACTGCAGACCAATCTGGTTTGGTTTCTG
AAAACCGGTGCAGGTAATAAACTGCCGCTGGCAGATGTTCGTGCAGCAATGCTGCTGCGTGCAA
ATAGCCACATGCATGGTGCAAGCGGTATTCGTCTGGAACTGATTAAACGCGCGGAAATCTTTCTG
AATGCCGGTGTTACCCCGTATGTTTATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCC
GCTGAGCTATATTACCGGTAGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCA
AAGAAATGGACGCACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAA
AGAAGGTCTGGCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTAT
GATACCCAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGGCACTGAATGG
TACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTGGGCAG
CAGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGGATGGTAAACA
TGATTATCGTGATGGTGAACTGATCCAGGATCGTTATAGCCTGCGTTGTCTGCCGCAGTATCTGG
GTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAATTGAGATTAACAGCGTTACC
GATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATCATGGTGGTAATTTTCTGGGTCAGTA
TGTTGGTATGGGTATGGATCATCTGCGCTATTATATCGGTGGCCTGGCAAAACATCTGGATGTTC
AGATTGCACTGCTGGCATCACCGGAATTTAGCAATGGTCTGCCTCCGAGTCTGGTGGGTAATCGT
GAACGTAAAGTTAATATGGGTCTGAAAGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCT
GACCTTTTATGGTAATAGTATTGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATA
TTAACAGCCAGGGTTATACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTGGCCAGAATTAT
GTTGCCATTGCCCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCA
TTATGATGCACGTGCCCAGCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTG
TTGGTCAGAAACCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGGATGA
ACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCGTTCAGGACA
TTCTGCCGAACCTGCAT (SEQ ID NO:15)

Polypeptide Sequence of AvPAL Variant No. 1002 (SEQ ID NO:16):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVARVARNGTLVSLTNNTDILQGIQAS
CDYINNAVESGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADVRAA
MLLRANSHMHGASGIRLELIKRAEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDPSF
KVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAMGV
HALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYRDGELIQ
DRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDH
LRYYIGGLAKHLDVQIALLASPEFSNGLPPSLVGNRERKVNMGLKGLQICGNSIMPLLTFYGN
SIADRFPTHAEQFNQNINSQGYTSATLARRSVDIGQNYVAIALMFGVQAVDLRTYKKTGHYD
ARAQLSPATERLYSAVRHVVGQKPSSDRPYIWNDNEQGLDE
HIARISADIAAGGVIVQAVQDILPNLH (SEQ ID NO:16)

Polynucleotide Sequence of AvPAL Variant No. 1008 (SEQ ID NO:17):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGGCAATA
GCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGCGCGTGTTGCCCGT
AATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTATTCAGGCCAGCTGTGA
TTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTGTTACCAGCGGTTTTGGTGGTA
TGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACTGCAGACCAATCTGGTTTGGTTTCTG
AAAACCGGTGCAGGTAATAAACTGCCGCTGGCAGATGTTCGTGCAGCAATGCTGCTGCGTGCAA
ATAGCCACATGCATGGTGCAAGCGGTATTCGTCTGGAACTGATTAAACGCGCGGAAATCTTTCTG
AATGCCGGTGTTACCCCGTATGTTTATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCC
GCTGAGCTATATTACCGGTAGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCA
AAGAAATGGACGCACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAA
AGAAGGTCTGGCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTAT
GATACCCAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGGCACTGAATGG
TACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTGGGCAG
CAGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGGATGGTAAACA
TGATTATCGTGATGGTGAACTGATCCAGGATCGTTATAGCCTGCGTTGTCTGCCGCAGTATCTGG
GTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAATTGAGATTAACAGCGTTACC
GATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATCATGGTGGTAATTTTCTGGGTCAGTA
TGTTGGTATGGGTATGGATCATCTGCGCTATTATATCGGTGGCCTGGCAAAACATCTGGATACCC
AGATTGCACTGCTGGCATCACCGGAATTTAGCAATGGTCTGCCTCCGAGTCTGGTGGGTAATCGT
GAACGTAAAGTTAATATGGGTCTGAAAGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCT
GACCTTTTATGGTAATAGTATTGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATA
TTAACAGCCAGGGTTATACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTGGCCAGAATTAT
GTTGCCATTGCCCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCA
TTATGATGCACGTGCCCAGCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTG
TTGGTCAGAAACCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGGATGA
ACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCGTTCAGGACA
TTCTGCCGAACCTGCAT (SEQ ID NO:17)

Polypeptide Sequence of AvPAL Variant No. 1008 (SEQ ID NO:18):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVARVARNGTLVSLTNNTDILQGI
QASCDYINNAVESGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADVR
AAMLLRANSHMHGASGIRLELIKRAEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDP
SFKVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAM
GVHALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYRDGE
LIQDRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGM

DHLRYYIGGLAKHLDTQIALLASPEFSNGLPPSLVGNRERKVNMGLKGLQICGNSIMPLLTFY
GNSIADRFPTHAEQFNQNINSQGYTSATLARRSVDIGQNYVAIALMFGVQAVDLRTYKKTGH
YDARAQLSPATERLYSAVRHVVGQKPSSDRPYIWNDNEQGLDE
HIARISADIAAGGVIVQAVQDILPNLH (SEQ ID NO:18)

Polynucleotide Sequence of AvPAL Variant No. 1009 (SEQ ID NO:19):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGGCAATA
GCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGCGCGTGTTGCCCGT
AATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTATTCAGGCCAGCTGTGA
TTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTGTTACCAGCGGTTTTGGTGGTA
TGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACTGCAGACCAATCTGGTTTGGTTTCTG
AAAACCGGTGCAGGTAATAAACTGCCGCTGGCAGATGTTCGTGCAGCAATGCTGCTGCGTGCAA
ATAGCCACATGCATGGTGCAAGCGGTATTCGTCTGGAACTGATTAAACGCGCGGAAATCTTTCTG
AATGCCGGTGTTACCCCGTATGTTTATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCC
GCTGAGCTATATTACCGGTAGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCA
AAGAAATGGACGCACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAA
AGAAGGTCTGGCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTAT
GATACCCAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGGCACTGAATGG
TACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTGGGCAG
CAGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGGATGGTAAACA
TGATTATCGTGATGGTGAACTGATCCAGGATCGTTATAGCCTGCGTTGTCTGCCGCAGTATCTGG
GTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAATTGAGATTAACAGCGTTACC
GATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATCATGGTGGTAATTTTCTGGGTCAGTA
TGTTGGTATGGGTATGGATCATCTGCGCTATGAAATCGGTCTGCTGGCAAAACATCTGGATGTTC
AGATTGCACTGCTGGCATCACCGGAATTTAGCAATGGTCTGCCTCCGAGTCTGGTGGGTAATCGT
GAACGTAAAGTTAATATGGGTCTGAAAGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCT
GACCTTTTATGGTAATAGTATTGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATA
TTAACAGCCAGGGTTATACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTGGCCAGAATTAT
GTTGCCATTGCCCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCA
TTATGATGCACGTGCCCAGCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTG
TTGGTCAGAAACCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGGATGA
ACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCGTTCAGGACA
TTCTGCCGAACCTGCAT (SEQ ID NO:19)

Polypeptide Sequence of AvPAL Variant No. 1009 (SEQ ID NO:20):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVARVARNGTLVSLTNNTDILQGI
QASCDYINNAVESGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADVR
AAMLLRANSHMHGASGIRLELIKRAEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDP
SFKVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAM
GVHALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYRDGE
LIQDRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGM
DHLRYEIGLLAKHLDVQIALLASPEFSNGLPPSLVGNRERKVNMGLKGLQICGNSIMPLLTFY
GNSIADRFPTHAEQFNQNINSQGYTSATLARRSVDIGQNYVAIALMFGVQAVDLRTYKKTGH
YDARAQLSPATERLYSAVRHVVGQKPSSDRPYIWNDNEQGLDEHIARISADIAAGGVIVQAV
QDILPNLH (SEQ ID NO:20)

Polynucleotide Sequence of AvPAL Variant No. 1010 (SEQ ID NO:21):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGGCAATA
GCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGCGCGTGTTGCCCGT
AATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTATTCAGGCCAGCTGTGA
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TGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACTGCAGACCAATCTGGTTTGGTTTCTG
AAAACCGGTGCAGGTAATAAACTGCCGCTGGCAGATGTTCGTGCAGCAATGCTGCTGCGTGCAA
ATAGCCACATGCATGGTGCAAGCGGTATTCGTCTGGAACTGATTAAACGCGCGGAAATCTTTCTG
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GCTGAGCTATATTACCGGTAGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCA
AAGAAATGGACGCACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAA
AGAAGGTCTGGCAATGATGAATGGCACCAGCGTTATGACCGGTATTGCAGCAAATTGTGTTTAT
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CAGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGGATGGTAAACA
TGATTATCGTGATGGTGAACTGATCCAGGATCGTTATAGCCTGCGTTGTCTGCCGCAGTATCTGG
GTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAATTGAGATTAACAGCGTTACC
GATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATCATGGTGGTAATTTTCTGGGTCAGTA
TGTTGGTATGGGTATGGATCATCTGCGCTATGAAATCGGTCTGCTGGCAAAACATCTGGATACCC
AGATTGCACTGCTGGCATCACCGGAATTTAGCAATGGTCTGCCTCCGAGTCTGGTGGGTAATCGT
GAACGTAAAGTTAATATGGGTCTGAAAGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCTGCT
GACCTTTTATGGTAATAGTATTGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATA
TTAACAGCCAGGGTTATACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTGGCCAGAATTAT
GTTGCCATTGCCCTGATGTTTGGTGTTCAGGCAGTTGATCTGCGTACCTACAAAAAAACCGGTCA
TTATGATGCACGTGCCCAGCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTG
TTGGTCAGAAACCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGGATGA
ACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCGTTCAGGACA
TTCTGCCGAACCTGCAT (SEQ ID NO: 21)

Polypeptide Sequence of AvPAL Variant No. 1010 (SEQ ID NO:22):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVARVARNGTLVSLTNNTDILQGI
QASCDYINNAVESGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADVR
AAMLLRANSHMHGASGIRLELIKRAEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDP
SFKVDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAM
GVHALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYRDGE
LIQDRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGM
DHLRYEIGLLAKHLDTQIALLASPEFSNGLPPSLVGNRERKVNMGLKGLQICGNSIMPLLTFY
GNSIADRFPTHAEQFNQNINSQGYTSATLARRSVDIGQNYVAIALMFGVQAVDLRTYKKTGH
YDARAQLSPATERLYSAVRHVVGQKPSSDRPYIWNDNEQGLDE
HIARISADIAAGGVIVQAVQDILPNLH (SEQ ID NO:22)

Polynucleotide Sequence of AvPAL Variant No. 1084 (SEQ ID NO:23):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCCATACCGGCAAT
AGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGTACGTGTTGCCCG
TAATGGCACCGCGGTTAGCCTGACCAATAATAAAGATATTCTGCAGCGTATTCAGGCCAGCTGT
GATTATATCAATAATGCAGTTGAAAAAGGTGAACCGATTTATGGTGTTACCAGCGGTTTTGGTGG
TATGGCAAATGTTGTAATTAGCCGTGAACAGGCAAGCGAACTGCAGACCAATCTGGTTTGGTTTC
TGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCAGATGTTCGTGCAGCAATGCTGCTGCGTGC
AAATAGCCACATGCGTGGTGCAAGCGGTATTCGTCTGGAACTGATTAAACGCATGGAAATCTTT

CTGAATGCCGGTGTTACCCCGTATGTTTATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGT
TCCGCTGAGCTATATTACCGGTAGCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATG
GCAAAGAAATGGACGCACCGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCAGCC
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TATGATACCCAGATTCTGACCGCAATTGCAATGGGTGTTCATGCACTGGATATTCAGGCACTGAA
TGGTACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTGGG
CAGCAGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGGATGGTAA
ACATGATTATATGGATGGTGAACTGATCCAGGATCGTTATAGCCTGCGTTGTCTGCCGCAGTATC
TGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAATTGAGATTAACAGCGTT
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CGTGAACGTAAAGTTAATATGGGTCTGAAAGGTCTGCAGATTTGCGGTAATAGCATTATGCCGCT
GCTGACCTTTTATGGTAATAGTATTGCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGA
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TCATTATGATGCACGTGCCCAGCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATG
TTGTTGGTAAAAAACCGAGCTCAGATCGTCCGTATATTTGGAATGATAATGAACAGGGTCTGGA
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ACATTCTGCCGCCGCTGCAT

Polypeptide Sequence of AvPAL Variant No. 1084 (SEQ ID NO:24):

MKTLSQAQSKTSSQQFSHTGNSSANVIIGNQKLTINDVVRVARNGTAVSLTNNKDILQRI
QASCDYINNAVEKGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADV
RAAMLLRANSHMRGASGIRLELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLD
PSFKVDFNGKEMDAPTALRQLNLSPLTLQPKEGLAMMNGTSVMTGIAANCVYDTQILTAIA
MGVHALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYMD
GELIQDRYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGM
GMDHLRYYIGLLAKHLDVQIALLASPEFSNGLPPSLVGNRERKVNMGLKGLQICGNSIMPLL
TFYGNSIADRFPTHAEQFNQNINSQGYTSATLARRSVDIFQNYVAIALMFGVQAVDLRTYKK
TGHYDARAQLSPATERLYSAVRHVVGKKPSSDRPYIWNDNEQGLDE
HIARISADIAAGGVIVQAVQDILPPLH (SEQ ID NO:24)

Polynucleotide Sequence of AvPAL Variant No. 967 (SEQ ID NO:25):

ATGAAAACCCTGAGTCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTACCGG
CAATAGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTGCGC
GTGTTGCCCGTAATGGCACCCTGGTTAGCCTGACCAATAATACCGATATTCTGCAGGGTA
TTCAGGCCAGCTGTGATTATATCAATAATGCAGTTGAAAGCGGTGAACCGATTTATGGTG
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TGCAGACCAATCTGGTTTGGTTTCTGAAAACCGGTGCAGGTAATAAACTGCCGCTGGCAG
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GTCTGGAACTGATTAAACGCGCGGAAATCTTTCTGAATGCCGGTGTTACCCCGTATGTTT
ATGAATTTGGTAGCATTGGTGCCAGCGGTGATCTGGTTCCGCTGAGCTATATTACCGGTA
GCCTGATTGGCCTGGACCCGAGCTTTAAAGTTGATTTTAATGGCAAAGAAATGGACGCAC
CGACCGCACTGCGTCAGCTGAATCTGAGTCCGCTGACCCTGCTGCCGAAAGAAGGTCTG
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ACAAATCAGAGCTTTCATCCGTTTATCCATAACAGCAAACCGCATCCGGGTCAGCTGTGG
GCAGCAGATCAGATGATTAGCCTGCTGGCCGGTAGCCAGCTGGTTCGTGATGAACTGGA

TGGTAAACATGATTATCGTGATGGTGAACTGATCCAGGATCGTTATAGCCTGCGTTGTCT
GCCGCAGTATCTGGGTCCGATTGTTGATGGTATTAGCCAGATTGCCAAACAAATCGAAAT
TGAGATTAACAGCGTTACCGATAACCCGCTGATTGATGTTGATAATCAGGCAAGCTATCA
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GCAGATCGTTTTCCGACCCATGCCGAACAGTTTAACCAGAATATTAACAGCCAGGGTTAT
ACCAGCGCAACCCTGGCACGTCGTAGCGTTGATATTGGCCAGAATTATGTTGCCATTGCC
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GCACGTGCCCAGCTGTCACCGGCAACCGAACGTCTGTATAGCGCAGTTCGTCATGTTGTT
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TGAACATATTGCACGTATTAGTGCAGATATTGCAGCCGGTGGTGTTATTGTTCAGGCCGT
TCAGGACATTCTGCCGAACCTGCAT (SEQ ID NO:25)

Polypeptide Sequence of Variant No. 967 (SEQ ID NO:26):

MKTLSQAQSKTSSQQFSFTGNSSANVIIGNQKLTINDVARVARNGTLVSLTNNTDILQGIQAS
CDYINNAVESGEPIYGVTSGFGGMANVVISREQASELQTNLVWFLKTGAGNKLPLADVRAA
MLLRANSHMRGASGIRLELIKRAEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDPSFK
VDFNGKEMDAPTALRQLNLSPLTLLPKEGLAMMNGTSVMTGIAANCVYDTQILTAIAMGVH
ALDIQALNGTNQSFHPFIHNSKPHPGQLWAADQMISLLAGSQLVRDELDGKHDYRDGELIQD
RYSLRCLPQYLGPIVDGISQIAKQIEIEINSVTDNPLIDVDNQASYHGGNFLGQYVGMGMDHL
RYYIGGLAKHLDVQIALLASPEFSNGLPPSLVGNRERKVNMGLKGLQICGNSIMPLLTFYGNS
IADRFPTHAEQFNQNINSQGYTSATLARRSVDIGQNYVAIALMFGVQAVDLRTYKKTGHYDA
RAQLSPATERLYSAVRHVVGQKPSSDRPYIWNDNEQGLDE
HIARISADIAAGGVIVQAVQDILPNLH (SEQ ID NO:26)

Expression vector pCK100900i:

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CCGGAGCGTATTTTTTGAGTTATCGAGATTTTCAGGAGCTAAGGAGGAACTAAAATGGA
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AGAATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAACTGCA
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GGATTCACTGGCCGTCGTTTTACAATCTAGAGGCCAGCCTGGCCATAAGGAGATATACAT
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CATATGAAAACCCTGAGCCAGGCACAGAGCAAAACCAGCAGCCAGCAGTTTAGCTTTAC
CGGCAATAGCAGCGCAAATGTGATTATTGGTAATCAGAAACTGACCATCAATGATGTTG
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GTTCAGGACATTCTGCCGTGTCTGCATTAAGGCCAAAC (SEQ ID NO:27)

EXAMPLE 1
PAL Gene Acquisition and Construction of Expression Vectors

[0230] *Anabaena variabilis* phenylalanine ammonia lyase (AvPAL) plasmid DNA was obtained and a synthetic gene encoding AvPAL was codon optimized for expression in *E. coli* and cloned into the *E. coli* expression vector pET16b to provide pET16b-AvPAL (SEQ ID NO:1). The AvPAL open reading frame (SEQ ID NO:2) was amplified by PCR using the oligonucleotides: PAL-pCK-F and PAL-pCK-R and subcloned into the expression vector pCK100900i (SEQ ID NO: 27).

Primer	Sequence 5' to 3'	SEQ ID NO:
PAL-pCK-F	CTAGAGGCCAGCCTGGCCATAAGGAGATATACAT ATGAAAACCCTGAGCCAGGCAC	SEQ ID NO: 28
PAL-pCK-R	GATGGTGATGGTGGCCAGTTTGGCCTTAATGCAG ACACGGCAGAATG	SEQ ID NO: 29

[0231] This plasmid construct was transformed into an *E. coli* strain derived from W3110. Directed evolution techniques generally known by those skilled in the art were used to generate libraries of gene variants from this plasmid construct (See e.g., US Pat. No. 8,383,346 and WO2010/144103).

EXAMPLE 2
High-Throughput (HTP) Growth and Assays

High-Throughput (HTP) Growth of PAL and PAL Variants

[0232] Transformed *E. coli* cells were selected by plating onto LB agar plates containing 1% glucose and 30μg/ml chloramphenicol. After overnight incubation at 37°C, colonies were placed into NUNC™ (Thermo-Scientific) the wells of 96-well shallow flat bottom plates filled with 180 μl/well LB supplemented with 1% glucose and 30μg/ml chloramphenicol. The cultures were allowed to grow overnight for 18-20 hours in a shaker (200 rpm, 30 °C, and 85% relative humidity; Kuhner). Overnight growth samples (20 μL) were transferred into Costar 96-well deep plates filled with 380μL

of Terrific Broth supplemented with 30μg/ml chloramphenicol. The plates were incubated for 135 minutes in a shaker (250 rpm, 30 °C, and 85% relative humidity; Kuhner). The cells were then induced with 40 μL of 10 mM IPTG in sterile water and incubated overnight for 20-24 hours in a shaker (250 rpm, 30°C, and 85% relative humidity; Kuhner). Two replicate cultures were combined, the cells were pelleted (4000 rpm x 20 min), the supernatants were discarded, and the cells were frozen at -80 °C prior to analysis.

Lysis of HTP Pellets

[0233] First, 500 μL of lysis buffer (20 mM Tris pH 7.5, 1 mM $MgSO_4$, 1 mg/ml lysozyme, and 0.5 mg/ml polymyxin B sulfate) were added to the cell pellets. The mixture was agitated for 1.5 h at room temperature, and pelleted (4000 rpm x 5 min) prior to use of the clarified lysates in the various HTP assays described herein. Analysis of these lysates by SDS-PAGE revealed the presence of an overexpressed protein at an apparent MW of ~60 kDa, consistent with the expected MW of PAL.

Analysis of Clarified Lysates

[0234] PAL variant activity was determined by measuring the formation of cinnamic acid as determined by the change in absorbance at 290 nm over time. For this assay, 100 μL of either 200 mM Tris/50 mM phenylalanine, pH 7.5, or 200mM sodium phosphate/50mM phenylalanine pH 7.0, 80 μL of water, and 20 μL of clarified lysate were added to the wells of a poly-acrylate 96-well plate (Costar #3635, Corning). The reactions were mixed briefly and the activity was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader.

HTP-Analysis of Clarified Lysates Pretreated with Protease

[0235] PAL variants were challenged with chymotrypsin and trypsin to simulate the environment of the lower intestine. First, 30 μL of protease mix (0.01 - 100 mg/ml chymotrypsin (C4129 Sigma Aldrich), 0.01 -100 mg/ml trypsin (T7409 Sigma Aldrich), 1 mM $CaCl_2$, and 1 mM HCl), 0- 30μL of 20 mM sodium taurocholate in 500 mM sodium phosphate pH 7.0, and 90 -120 μL of clarified lysate were added to the wells of a 96-well round bottom plate (Costar #3798, Corning). The plates were sealed and incubated at 37 °C, 400 rpm, 1" throw for 1h prior to analysis. For the assay, 100 μL of either 200 mM Tris/50 mM phenylalanine pH 7.5 or 200 mM sodium phosphate/50 mM phenylalanine pH 7.0 and 100 μL of the protease treated lysate were added to the wells of a poly-acrylate 96-well plate (Costar #3635, Corning). The reactions were mixed briefly and the activity was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader. The results are provided in the following Tables.

HTP-Analysis of Clarified Lysates Pretreated with Acid

[0236] In this assay, PAL variants were challenged under acidic conditions, in order to simulate the environment of the stomach. First, 20 µL of 1M sodium citrate (pH 3.7-4.5) and 30 µL of water or 50 µL of 400 mM sodium citrate pH 3.7-4.8, and 50 uL of clarified lysate were added to the wells of a 96-well round bottom plate (Costar #3798, Corning). The plate was sealed and incubated at 37 °C, 400 rpm, 1" throw for 1h prior to analysis. For the assay, 100 µL of either 200 mM Tris, 50 mM phenylalanine pH 7.5 and 80 µL 1M Tris pH 7.5 or 200 mM sodium phosphate/50 mM phenylalanine pH 7.0 and 80µL of 1.0 M sodium phosphate pH7.0, and 20 µL of the acid-treated lysate were added to the wells of a poly-acrylate 96-well plate (Costar #3635, Corning). The reactions were mixed briefly, and the activity was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader. The results are provided in the following Tables.

HTP Analysis of Clarified Lysates Pretreated with Pepsin

[0237] In additional assays, PAL variants are challenged with acidic conditions and pepsin to further test the variants under conditions that mimic the gastric environment. First, 50 µL of 0.01-100 mg/ml pepsin in 400 mM sodium citrate pH 1.5-4, and 50 µL of clarified lysate are added to the wells of a 96-well round bottom plate (Costar #3798, Corning). The plate is sealed and incubated at 37 °C, 400 rpm, 1" throw for 1-12h prior to analysis. For the assay, 100 µL of either 200 mM Tris/50 mM phenylalanine pH 7.5 and 80 µL 1M Tris pH 7.5 or 200 mM sodium phosphate/50 mM phenylalanine pH 7.0, and 20 µL of acid-treated lysate are added to the wells of a poly-acrylate 96-well plate (Costar #3635, Corning). The reactions are mixed briefly, and the activity is determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader.

Table 2-1.
Relative Activity of PAL Variants When Unchallenged (U-C), Protease-Challenged (P-C), and Acid-Challenged (A-C).

Variant #	U-C	P-C	A-C	Amino Acid Differences Relative to SEQ ID NO:3
1	+	+	+	A39V/A91V/Y158H/S180A/N290G/A394V/S399N/ N474Q/K522Y/T524S
2	+	+	++	A39V/A91V/Y158H/A256G/A394V/P404A/N474Q
3	+	++	++	A39V/A91V/S180A/A394V/K522F/T524S
4	+	+	++	A39V/A91V/Y158H/T243I/A256G/S399N/P404A/L407 V/N474Q
5	+	+	++	E308Q
6	+	++	+	N400K
7	+	+	+	L364Q
8	+	+	++	A256G/N290G/P404A/L407V/N474Q/K522F
9	+	+	++	Y158H/S180A/A394V/T463N/N474Q/T524S

Table 2-1.
Relative Activity of PAL Variants When Unchallenged (U-C), Protease-Challenged (P-C), and Acid-Challenged (A-C).

Variant #	U-C	P-C	A-C	Amino Acid Differences Relative to SEQ ID NO:4
10	+	+	++	A39V/A91V/Y158H/S180A/K195E/A256G/S399N/ L407V/Y459F/T463N/K522N/T524S
11	+	+	++	A39V/A91V/Y158H/S180A/K195E/A256G/ N290G/S399N/Y459F/T463N
12	+	++	++	A39V/A91V/Y158H/S180A/K195E/T243I/A394V
13	+	++	++	A39V/A91V/Y158H/K195E/T243I/A256G/A394V/ S399N/N474Q/K522Y/T524S
14	+	++	++	A39V/A91V/Y158H/S180A/K195E/I245L/S399N/ L407V/Y459F/T463N/N474Q/T524S
15	+	++	+	H307M
16	+	+	++	A39V/A91V/Y158H/S180A/T243L/A394V/S399N/ T463N/K522F
17	+	+	++	A91V/N474Q
18	++	+	++	S180A/K195E
19	++	+	++	A91V/Y158H/T243I/A256G/N290G/S399N/L407V/ Y459F/ T463N/N474Q/K522N/T524S
20	++	++	++	A39V/Y158H/S180A/S399N
21	++	++	++	A91V/N270K
22	++	+++	++	H307G
23	++	++	+	N453G
24	++	++	++	H307Q
25	++	++	+	L257W
26	++	+	+++	A91V/Y158H/I245L/A256G/S399N/Y459F/T463N/ K522Y/T524S
27	++	++	+	F443H
28	++	++	++	A91V/Y158H/S180A
29	++	++	+	A91Y/ Y158H/ K195E/ S399N
30	++	++	+++	A39V/ A91V/ A256G/ N290G/ A394V/ S399N/ P404A/ L407V/K522Y/ T524S
31	++	++	+	L257A
32	++	+	+++	Y158H/K195E/T243L/A256G/A394V/S399N/ L407V/N474Q/ K522F/T524S
33	++	++		L349M
34	+++	+++	+	I326F
35	+++	+++	+++	T460G/ P528L

1. Relative activity was calculated as activity of the variant/activity of SEQ ID NO:4 (encoded by SEQ ID NO:3).
2. Variant # 22 has the polynucleotide sequence of SEQ ID NO:7 and polypeptide sequence of SEQ ID NO:8, and Variant # 30 has polynucleotide sequence of SEQ ID NO:5 and polypeptide sequence of SEQ ID NO:6.
3. + = 0.1 to 1.0 relative activity over wild-type AvPAL;
++ = >1.0 to 2.0 relative activity over wild-type AvPAL; and
+++ = >2.0 relative activity over wild-type AvPAL.

Table 2-2. Relative Activity of PAL Variants Unchallenged (U-C), Protease-Challenged (P-C), and Acid-Challenged (A-C).				
Variant #	**U-C**	**P-C**	**A-C**	**Amino Acid Differences – Relative to Wild-Type AvPAL (SEQ ID NO:4)**
36	++	+++	++	A39V/A91V/N290G/H307G/L407V/ T524S
37	++	+++	++	A39V/A91V/N290G/H307G/L407V
38	+	++	+	A39V/A91V/A256G/N290G/H307G/S399N/P404A/ L407V/K522Y/T524S
39	++	+	++	A39V/A91V/A256G/N290G/S399N/P404A/L407V/K522 Y
40	+	++	+	A39V/A256G/N290G/H307G/P404A/L407V
41	+	++	+	A39V/A91V/A256G/N290G/H307G/P404A/L407V/T524S

1. Relative activity was calculated as activity of the variant/activity of Variant No. 30.
2. Variant # 36 has polynucleotide sequence of SEQ ID NO:9 and polypeptide sequence of SEQ ID NO:10.
3. + = >1.0 to 3.0 relative activity over Variant 30;
   ++ = >3.0 to 10 relative activity over Variant 30; and
   +++ = >10 to 35 relative activity over Variant 30.

Table 2-3. Relative Activity of PAL Variants Unchallenged (U-C), Protease-Challenged (P-C), and Acid-Challenged (A-C).				
Variant #	**U-C**	**A-C**	**P-C**	**Mutations (Relative to Variant No. 36)**
42	+	+	+	T54K/G59R/S73K/R305M/C503Q/Q521K/C565P
43	+	+++	++	T54K/G59R/R305M/C503Q/Q521K/C565N
44	++	+++	++	G59R/C503Q
45	++	+++	++	K32P/G59R/S73K/Q240W/C503Q/C565N
46	+	+	+	K32P/G59R/S73K/Q240W/C565P
47	+	+++	++	K32P/T54K/S73K/R305M/C503Q/Q521K/C565N
48	+++	+++	++	Y304H/D353A
49	++	+++	+++	S73K/D353A
50	+	+	++	A112C/S546R
51	++	+	++	S73K/Q240W/Y304H
52	+	+	++	R134Q/Q240W/Y304H/D353A/E509L

1. Relative activity was calculated as activity of variant/ activity of Variant No 36.
2. Variant # 42 has the polynucleotide sequence of SEQ ID NO:11 and a polypeptide sequence of SEQ ID NO:12. Variant # 43 has the polynucleotide sequence of SEQ ID NO: 13 and a polypeptide sequence of SEQ ID NO:14.
3. + = > 0.5 to 1.5 relative activity over Variant 36;
   ++ = > 1.5 to 3 relative activity over Variant 36; and
   +++ = > 3 to 10 relative activity over Variant 36.

Table 2-4.
Relative Activity of PAL Variants Unchallenged (U-C), Protease-Challenged (P-C), and Acid-Challenged (A-C).

Variant #	U-C	P-C	A-C	Mutations Relative to Variant #30
53	+	+	+	D303R
54	+	+	++	E308A
55	+	+	+	E308D
56	++	++	+	G256A
57	+	++	+	H307A
58	+	+++	+	H307D
59	+	++	+	H307E
60	+	++	+	H307F
61	+	+++	+	H307G
62	+	+	+	H307I
63	+	+	+	H307L
64	++	+++	++	H307M
65	+	++	+	H307N
66	++	++	++	H307R
67	+	+	+	H307Y
68	+	+++	+	R305L
69	+	+++	+	R305M
70	+	+++	+	R305Q
71	++	++	+	V91A/G256A
72	+	++	+	Y304H
73	+	+	+	Y304W
74	+	+	+	C503K
75	+	+	+	C503Q
76	+	+	+	C565A
77	+	+	+	C565G
78	+	+	+	C565I
79	+	+	+	C565K
80	+	+	+	C565L
81	+	++	+	C565N
82	+	+	+	C565P
83	+	+	+	C565T

1. Relative activity was calculated as activity of variant/ activity of Variant No. 30 (Variants 53-73) or SEQ ID NO:4 (encoded by SEQ ID NO:3) (Variants 74-83.)

2. + = > 0.5 to 1.5 relative activity over Variant 30; ++ = > 1.5 to 3 relative activity over Variant 30; and +++ = > 3 to 10 relative activity over Variant 30.

Table 2-5.
Relative Activity of PAL Variants Unchallenged (U-C), Protease-Challenged (P-C), and Acid-Challenged (A-C).

Variant #	U-C	P-C	A-C	Mutations Relative to Variant 42
42	+	+	+	
1011	+	+	+	R59G/R134Q/Q240R/K521Q/P564L
1012	+	+	++	R59G/P564M
1013	+	+	+	R59G/Q240W/E509L/K521Q/P564M
1014	++	++	+	Q240W/Y304H/D353N/E509L/K521S/P564L
1015	+	++	+	R59G/R134Q/Q240R/K521Q/P565N
1016	+	+++	+	Y304H/D353A/E509L
1017	+	++	+	R59G/R134Q/Y304H/D353N/K521Q
1018	+	+++	+	R59G/R134Q/Q240R/K521S
1019	+	+	+	R134Q/Y304H/D353A/K521S/P565N
1020	+	+	+	R59G/R134Q/Q240R/Y304H/D353A/K521Q/P564M
1021	++	+++	++	Q240W/D353A/E509L/K521Q
1022	+	++	+	R134Q/Q240W/E509L/K521S
1023	++	++	++	R134Q/Q240R/D353A/K521Q/P564G
1024	+	+	+	R134Q/K521Q
1025	+	+++	+	K521Q
1026	+	+++	+	R134Q/D353A/K521S/P564M
1027	+	+++	+	R59G/R134Q/Q240W/D353A
1028	++	++	+	R59G/K521S
1029	+	++	+	R59G/D353A
1030	+	+	+	Q240R/D353A/K521S
1031	+	+	+	R59G/D353A/E509L/P564L
1032	+	+	+	R59G/D353A/K521Q
1033	+	+	+	R59G/R134Q/D353A/E509L/K521Q
1034	+	+	+	Q240R/Y304H/E509L
1035	+	+	+	R59G/Q240R/D353A/E509L
1036	+	+	+	R59G/R134Q/E509L
1037	+	+	+	Q240W/Y304H/E509L/K521S
1038	+	+	+	Q240W/D353A/E509L/K521S/P564L/P565N
1039	+	+	+	R59G/Q240R/D353A/P564M/P565N
1040	+	+	+	R134Q/D353A/K521S
1041	+	+	+	R134Q/Q240W/Y304H/D353A/P564L
1042	+	+	+	Q240R/E509L/P565N

Table 2-5.
Relative Activity of PAL Variants Unchallenged (U-C), Protease-Challenged (P-C), and Acid-Challenged (A-C).

Variant #	U-C	P-C	A-C	Mutations Relative to Variant 42
1043	+	+	+	Q240R/K521S/P564M/P565N
1044	+	+	+	R134Q/D353A/P564L
1045	+	+	+	R59G/R134Q
1046	+	+	+	R59G/Q240R
1047	+	+	+	Y304H/E509L
1048	+	+	+	R134Q/Q240R/D353A/E509L/K521Q/P564L
1049	+	+	+	Q240W/E509L/P564L/P565N
1050	+	+	+	R59G/E509L/P564L
1051	+	+	+	R134Q/D353A/E509L/P564L
1052	+	+	+	R59G/D353A/P565N
1053	+	+	+	Q240R/E509L
1054	+	+	+	Q240R
1055	+	+	+	D353A/K521Q
1056	+	+	+	Q240W/D353A/E509L
1057	+	+	+	F18H/L47A/L214Q/E540D
1058	+	+	+	F18H/L47A/L214Q/E308K/F450Y/S546R
1059	+	+	+	F18H/L47A/F450Y/P528L/S546R
1060	+	+	+	L214Q/E308Q/T460G
1061	+	+	+	F18H/F450Y/E540D
1062	+	+	+	P528L/S546R
1063	+	+	+	F18H/L214Q/F450Y
1064	+	+	+	E308Q/F450Y/R467G
1065	+	+	+	F18H/E540D
1066	+	+	+	L47A/L214Q/S546R
1067	+	+	+	F18H/L214Q/E308Q
1068	+	+	+	L47A/L214Q/E308Q
1069	+	+	+	F18H/L47E/L214Q/R467G/E540D/S546R
1070	+	+	+	L47A/F450Y
1071	+	+	+	L47A/L214Q
1072	+	+	+	F18H/L47A/L214Q/S546R
1073	+	+	+	F18H/L214Q
1074	+	+	+	L214Q/F450Y/P528L
1075	+	+	+	F18H/L214Q/E308Q/F450Y/R467G

Table 2-5.
Relative Activity of PAL Variants Unchallenged (U-C), Protease-Challenged (P-C), and Acid-Challenged (A-C).

Variant #	U-C	P-C	A-C	Mutations Relative to Variant 42
1076	+	+	+	L47A/L214Q/E540D
1077	+	+	+	L214Q/E540D
1078	+	+	+	F18H/L47A/E308Q/S546R
1079	+	+	+	F18H
1080	+	+	+	F18H/L47A/F450Y/S546R
1081	+	+	+	F18H/L47A/L214Q/F450Y
1082	+	+	+	F18H/L47A
1083	+	+	+	L47A
1084	+	+	+	F18H/L47A/L214Q
1085	+	+	+	L214Q/S546R
1086	+	+	+	F18H/L214Q/R467G/S546R
1087	+	+	+	F18H/L47A/L214Q/E308Q
1088	+	+	+	F18H/L214Q/T460G
1089	+	+	+	F18H/L47A/F450Y
1090	+	+	+	F18H/L214Q/E540D

1. Relative activity was calculated as activity of variant/ activity of Variant No. 42
2. - < 0,5 relative activity over Variant No. 42; + = > 0.5 to 1.5 relative activity over Variant No. 42; ++ = > 1.5 to 3 relative activity over Variant No.42.

EXAMPLE 3

Assays to Determine Protein Aggregation of PAL Variants

[0238] The propensity of the PAL variants to aggregate is determined using the ProteoStat® Protein Aggregation Assay kit (Enzo), according to the manufacturer's instructions. Briefly, purified PAL at 0-100 μM is mixed with ProteoStat® detection reagent (1:2000) and analyzed via flow cytometry. Samples are assessed for fluorescence, consistent with the ProteoStat® Aggregation Standards, as known in the art (See e.g., Bershtein et al., Mol.Cell,, 133-144 [2013]).

EXAMPLE 4

Lyophilized Lysates from Shake Flask (SF) Cultures

[0239] Selected HTP cultures grown as described above were plated onto LB-agar plates with 1% glucose and 30 μg/ml chloramphenicol and grown overnight at 37 °C. A single colony from each culture was transferred to 50 ml of LB with 1% glucose and 30 μg/ml chloramphenicol. The cultures were grown for 18 h at 30 °C, 250 rpm, and subcultured at a dilution of approximately 1:10 into 250 ml of Terrific Broth with 30 μg/ml of chloramphenicol, to a final OD600 of 0.2. The cultures were incubated for 135 minutes at 30°C, 250 rpm, to an OD600 of 0.6 and induced with 1 mM of IPTG. The induced cultures were incubated for 20 h at 30°C, 250 rpm. Following this incubation period, the cultures were centrifuged 4000 rpm x 10 min. The supernatant was discarded, and the pellets were resuspended in 30 ml of 50 mM sodium phosphate pH 7.5. The cells were pelleted (4000 rpm x 10 min), resuspended in 12 ml of 50 mM sodium phosphate pH 7.5, and lysed using a One Shot Cell Disruption system (Constant Systems) at 17,000 psi. Lysate was pelleted (10,000 rpm x 30 min) and the supernatant was frozen and lyophilized to generate an enzyme-containing powder.

Purification of PAL From Shake Flask Cultures

[0240] PAL Variant No. 42 was grown in shake flask cultures to saturation, as described above. Saturated cultures were pelleted by centrifugation (4000 rpm x 20 min) and the cell pellets were stored at -80 °C prior to purification. The cell pellets were thawed at room temperature and resuspended in 25 mM Tris, pH 8 with 130 mM NaCl at 5 mL of buffer/ g of cells. The sample slurry was lysed using a microfluidizer with a pressure setting of 110 psi. The resulting lysate was clarified by centrifugation at 10,000 rpm for 1 hour, followed by filtration through a 0.2 μm PES filter (Millipore).

[0241] After filtration, the resulting lysate was heated at 70-85 °C for 1.5-2 hours in the presence or absence of 10 mM Phe. The lysate was removed from the heat and clarified by centrifugation at 10,000 rpm at 4 °C for 1 hour. The supernatant containing soluble PAL was then filtered through a 0.2 μm PES filter prior to loading onto a chromatography column.

[0242] The heat-treated, filtered lysate, containing 80-100 mg of total protein, was diluted two-fold using 25 mM Tris, pH 8 with 1.2 M ammonium sulfate. The sample was loaded on to a HiPrep 16/10 Phenyl FF (hi sub) column (GE Healthcare) pre-equilibrated with the 25 mM Tris, pH 8 with 0.6M ammonium sulfate. Following sample loading, the column was washed with three column volumes of the same buffer, followed by a linear gradient of 0.6 M – 0 M ammonium sulfate in 25 mM Tris, pH 8 for one column volume. Tightly-bound PAL was eluted off the column using an isocratic elution with 25 mM Tris, pH 8 for three column volumes. Fractions containing active and pure PAL were pooled.

[0243] The purified PAL from the phenyl column was buffer-exchanged into 0.5 M Tris, pH 8.5 and concentrated. The concentrated PAL was analyzed by SDS-PAGE and found to be present in a band

at ~60 kDa. The purified PAL samples were filtered using a 0.45 μm PES filter and were stored at -80 °C until ready for use.

EXAMPLE 5

Characterization of Purified PAL and PAL Variants

[0244] In this Example, assays conducted to characterize wild-type and variant PALs are described.

Tolerance to Acidic pH:

[0245] Lyophilized powders containing PAL variants were dissolved at 2 g/L in 20 mM sodium phosphate pH 7.0. Then, 50 μL of the enzyme solutions were mixed with 50 μL of 400 mM citric acid (pH 4.0-5.2) or 100 mM sodium phosphate and reactions were incubated at 37 °C for 1h at 400 rpm (1" throw). Then, 20 μL of the solution were briefly mixed with 80 μL of 1M sodium phosphate pH 7.0 and 100 μL of 200 mM Tris/50 mM phenylalanine pH 7.5. The enzymatic activity under acidic conditions was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader. The results are shown Figure 2. As indicated in Figure 2, Variant Nos. 30 and 36 maintained more activity after being incubated at pHs approximately 4 to 4.8, compared to the wild-type PAL.

Determination of K_M:

[0246] To evaluate whether the mutations in the variant PALs had altered the affinity of the PAL variants for phenylalanine, the Michaelis constant for the wild-type enzyme and Variant 36 were determined. First, 100 μL of 15 μg/ml PAL in 100 mM Tris pH 8.0, and 100 μL of 0-32 mM phenylalanine in 100 mM Tris, pH 8.0, were added to the wells of a poly-acrylate 96-well plate (Costar #3625, Corning). The reaction was mixed briefly and initial rates were determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader. The K_M for each tested PAL variant was determined by fitting the data to a Lineweaver-Burke plot, as known in the art. The results are shown in Figure 3. As shown, the K_M was 74 μM for the wild-type enzyme and 60 μM for Variant 36.

Amino Acid Specificity:

[0247] Some phenylalanine ammonia lyases demonstrate activity against tyrosine and/or histidine in addition to phenylalanine. To evaluate if the mutations present in the PAL variants had altered the specificity of the PAL variants for phenylalanine, the activities of wild-type enzyme and Variant 36

on these three amino acids were assessed. First, 100 µL of 5g/L of PAL-containing lyophilized powder in 10 mM sodium phosphate pH 7.0, and 100 µL of 50 mM phenylalanine or histidine or 2.5 mM tyrosine in 200 mM sodium phosphate pH 7.5 were added to the wells of a poly-acrylate 96-well plate (Costar #3635, Corning). The solutions were mixed briefly and initial rates were determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader. The results are shown in Figure 4. As indicated, no detectable activity was observed for either the WT enzyme or Variant No. 36 with either histidine or tyrosine, indicating that these enzymes are specific for phenylalanine.

Resistance to Porcine and Bovine Proteases:

[0248] PAL variant samples prepared as described in Example 4, were dissolved at 2 g/L in 100 mM sodium phosphate pH 7.0. Porcine trypsin and bovine chymotrypsin (100 mg each) were dissolved in 2 ml of 100 mM sodium phosphate pH 7.0, and serially diluted 2-fold eleven times in 100 mM sodium phosphate. Then, 80 µL of the PAL variant enzyme solutions were mixed with 20 µL of the trypsin and chymotrypsin solution. The reaction mixtures were incubated at 37 °C for 1h at 400 rpm (1" throw). Then, 20 µL of the reaction was mixed with 80 µL of water and 100 µL of 100 mM sodium phosphate, 50 mM phenylalanine pH 7.0. Each solution was mixed briefly, and the activity was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader. The results are shown in Figure 2. As indicated in this Figure, all of the tested variants showed improved protease resistance, as compared to the wild-type PAL, with Variant No. 36 being the most stable towards proteolysis.

Resistance to Human Proteases:

[0249] As described above, some evolved PAL variants were screened against porcine trypsin and bovine chymotrypsin to assess their resistance to proteolysis by enzymes present in the gastrointestinal tract. Some of the evolved PAL variants were also tested using human enzymes, to confirm that they are resistant to the human homologues of the porcine or bovine enzymes. In these assays, lyophilized powders of WT PAL and Variant No. 36 (2.4 g/L in 100 mM sodium phosphate, pH 7.0) were incubated with human chymotrypsin (Athens Research) 0-80 BTEE units/ml or human trypsin (ProSpec) (0-10,000 BAEE units/ml) at 37 °C for 2 h. Then, 100 µL of the mixtures were added to the wells of a poly-acrylate 96- well plate (Costar #3635, Corning), followed by the addition of 100 µL of 50 mM phenylalanine, 200 mM sodium phosphate pH 7.0. The solution was mixed briefly and initial rates were determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices)

absorbance microplate reader. . The results are shown in Figure 5. As shown in Figure 5, Variant No. 36 was more stable than the wild type PAL enzyme.

Resistance to Crude Pancreatic Extract:

[0250] The evolved PAL variants were also tested to determine their resistance to pancreatic enzymes. Lyophilized powders of WT PAL, Variant No. 36, Variant No. 42, and Variant No. 43 lyophilized powders (prepared as described in Example 4; 12 g/L in 50 mM potassium phosphate pH 6.8) were mixed 1:1 with porcine pancreatin (4x Sigma-Aldrich, St. Louis, MO), and incubated at 37 °C with shaking (400 rpm, 1" throw) for up to 23 h. At the indicated time points, a 10 µL aliquot of the reactions was added to 190 µL of 50 mM phenylalanine, 190 mM sodium phosphate pH 7.0, in the wells of a poly-acrylate 96- well plate (Costar #3635, Corning). The reaction was mixed briefly and initial rates were determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader. The results are shown in Figure 6. As shown in Figure 6, Variant No. 36, Variant No. 42, and Variant No. 43 all showed significant stability under these assay conditions, as compared to the wild-type PAL enzyme.

Impact of Intestinal Detergents:

[0251] The evolved PAL variants were also tested for their susceptibility to proteolysis in the presence or absence of intestinal bile acids and fatty acids, to assess whether these acids impact their stability. Lyophilized powders containing Variant No. 36 (prepared as described in Example 4) were dissolved at 50 µg/ml in 0-16 mM sodium taurocholate, 100 mM sodium phosphate, pH 7.0. Porcine trypsin and bovine chymotrypsin (80 mg each) were dissolved in 2 ml of 100 mM sodium phosphate pH 7.0, and serially diluted 2-fold eleven times in 100 mM sodium phosphate. For the assay, 50 µL of the PAL solutions were mixed with 50 µL of the protease solution. The mixtures were incubated at 37 °C for 1h at 400 rpm (1" throw). Then, 50 µL of the mixtures were mixed with 150 µL of 200 mM sodium phosphate, 50 mM phenylalanine pH 7.0. Each reaction was mixed briefly, and the activity was determined by tracking the absorbance at 290 nm over time (every 12-20s over 5-20 min) using a SpectraMax® Plus^{384} or a SpectraMax® 190 (Molecular Devices) absorbance microplate reader. The results are shown in Figure 7. As shown in this Figure, additional sodium taurocholate increases the susceptibility of Variant No. 36 to proteolysis.

EXAMPLE 6

Intestinal Stability of Variant PAL

[0252] To assess the stability and activity of PAL variants as they transit through an animal gut, mice were gavaged with purified enzyme variants. Healthy C57Bl/6 mice, 10-12 weeks old and weighing 20-26 g, were maintained in a metabolic cage and fasted for 15h. Water was provided *ad libitum*. Following the overnight fast, animals were gavaged using a 21-gauge gavage needle with a mixture of 0.3 ml of 0.5 M Tris-HCl pH 8.5, and 8 mg/ml in 0.5 M Tris-HCl pH 8.5, WT PAL (prepared as described in Example 4) or 8 mg/ml in 0.5 M Tris-HCl pH 8.5Variant No. 42 (prepared as described in Example 4). At 0.5, 2, or 6 h post-gavage, the animals were decapitated, plasma was collected using green-top capillary blood collection tubes (Ram Scientific), and the contents of the stomach, duodenum (~1-8 cm from the stomach), jejunum (~10-18 cm from the stomach), ileum (~8 cm above the cecum), and colon (~5 cm below the cecum) were collected. The weight of these contents was recorded and the contents were stored at -80 ˚C prior to analysis.

[0253] Stomach or intestinal contents were diluted 4X with 100 mM sodium phosphate pH 7.0, mixed briefly, and centrifuged at 14,000 rpm x 2 min. The supernatants were transferred to a 350 µL, 0.45 µM, AcroPrep™ Advanced 96-well filter plate (Pall Corp), and particulates were removed via vacuum filtration. The clarified filtrate was assessed for enzymatic activity as described in the previous Examples and for the presence of intact PAL protein by SDS-PAGE. The results indicated that enzymatic activity in the duodenum and jejunum appeared to be higher for the evolved PAL variants, as compared to the wild-type PAL enzyme and negative control.

EXAMPLE 7

Plasma Phenylalanine Levels

[0254] Plasma samples collected from the mice described in Example 6 were evaluated to determine the quantity of phenylalanine present in the bloodstreams of the tested mice. Mouse plasma (50 µL) was combined with 250 µL of acetontrile containing 0.6 mM of dl-phenylalanine (Ring D_5) (i.e., an isotopically labeled version of phenylalanine comprising deuterium rather than hydrogen bonded with the aromatic ring carbons; Cambridge Isotope Laboratories). The samples were mixed at RT for 5 min, centrifuged at 3200 x g for 10 min at 4 ˚C and the supernatants were transferred to a plate for sample analysis. For the analysis, 10 µL of each sample was injected into a3200 QTRAP® LC/MS/MS system (AB Sciex) across a DISCOVERY® C_{18} column (150 x 2.1 mM, 5µm beads) (Supelco, now Sigma-Aldrich), eluting with 0.1 % formic acid in water (A) and acetonitrile (B). Samples were eluted across a 5 min gradient (t=0, 97% A; 3 min, 50% A; 3.5 min, 5% A; 4 min, 97%A; 5 min, 95% A) looking for a transition of 166 to120 for endogenous phenylalanine and 171 to 125 for the isotopically labeled standard. The results indicated that the plasma phenylalanine levels were lower at the 30

minute time-point in samples from mice that were given the evolved PAL variant (i.e., Variant No. 42), as compared to the wild-type PAL enzyme and negative control.

EXAMPLE 8

Therapeutic Function of Variant PAL

[0255] To assess whether PAL variants reduce serum Phe levels *in vivo*, a mouse model of PKU was used. In these experiments, the PAL proteins were gavaged into affected animals. First, a consistent baseline Phe level was established in the mice by removing Phe from their diets for three days followed by injection of known quantity of Phe-containing solution. Three- to six-month old homozygous PAH enu-2 mice with a C57Bl/6 background (See, McDonald et al., Proc. Natl. Acad. Sci. USA 87:1965-1967 [1990]) were transferred to a phenylalanine-free diet (TD.97152, Harlan) with 0.03 g/L of Phe provided in their drinking water for 72 h. Prior to initiating treatment at time = 0 h, mice were injected with 0.15 mg/g of body weight Phe (from a 10 g/L solution of Phe in water). Fifty-five minutes post-injection, approximately 20 µL of blood was collected by tail-vein puncture and spotted onto filter paper. Subsequently, at times 1 h-, 3 h-, and 5 h- post injection the mice were gavaged with 0.3 ml of 50-100 g/L of WT AvPAL, WT AvPAL plus aprotinin, BSA, or Variant No. 42. At 6 h-, 7 h-, and 9 h-post injection, additional blood spots were collected on filter paper. The blood spots were dried and stored at -20 °C prior to LC-MS/MS analysis for Phe and Tyr levels using methods known in the art (See, Chase et al., Clin. Chem., 39:66-71 [1993]).

[0256] The results are shown in Figure 8. As indicated in this Figure, gavage with inactive protein (BSA), led to increased serum Phe levels . In contrast, treatment with proteolytically susceptible WT AvPAL resulted in constant Phe-levels, while the same protein, combined with the protease inhibitor aprotinin resulted in a significant drop in Phe-levels. The results also show that administering the engineered-PAL Variant No. 42 resulted in decreased serum Phe levels in the absence of protease inhibitors.

EXAMPLE 9

Deimmunization of PAL

[0257] In this Example, experiments conducted to identify diversity that would remove T-cell epitopes from PAL are described.

Identification of Deimmunizing Diversity:

[0258] To identify amino acids that, when mutated, could remove T-cell epitopes, computational methods were used to identify PAL sequences predicted to elicit a T-cell response. In parallel,

experimental searches for allowable, non-deleterious mutations were also conducted, particularly for amino acids that maintain protein activity in an unchallenged assay (e.g., in the assays described in Example 2). Active variants were then analyzed for the effect of the mutations on the predicted immunogenicity.

Computational Identification of Putative T-cell Epitopes in a Variant AvPAL:

[0259] Putative T-cell epitopes in a AvPAL Variant No. 36 were identified using the Immune Epitope Database (IEDB; Immune Epitope Database and Analysis Resource website) tools, as known in the art and proprietary statistical analysis tools (See e.g., iedb.org and Vita et al., Nucl. Acids Res., 38(Database issue):D854-62 [2010]. Epub 2009 Nov 11]). The AvPAL variant was parsed into all possible 15-mer analysis frames, with each frame overlapping the last by 14 amino acids. The 15-mer analysis frames were evaluated for immunogenic potential by scoring their 9-mer core regions for predicted binding to eight common Class II HLA-DR alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301, and DRB1*1501) that collectively cover nearly 95% of the human population (See e.g., Southwood et al., J. Immunol., 160:3363-3373 [1998]), using methods recommended on the IEDB website. Potential T-cell epitope clusters contained within the variant AvPAL (i.e., sub-regions contained within the variant AvPAL which have an unusually high potential for immunogenicity) were identified using statistical analysis tools, as known in the art. The identified T-cell epitope clusters were screened against the IEDB database of known epitopes and the GenBank protein database. These screens identified 10 (ten) putative T-cell epitopes in the variant AvPAL Variant No. 36. These epitopes are referred to as TCE-I, II, III, IV, V, VI, VII, VIII, IX, and X, below.

Design of Deimmunizing Libraries:

[0260] First, a combinatorial library containing all neutral, and beneficial mutations identified from the rounds of directed evolution used to create variant AvPALs, at the 10 putative T-cell epitope regions identified as described above was developed. The effects of these mutations on the predicted binding to the eight common Class II HLA-DR alleles were analyzed. Multiple mutations were predicted to remove or reduce TCE-I, II, VI, VII. These mutations were combined into a combinatorial library. Libraries were then designed using saturation mutagenesis to mutagenize every single amino acid within the remaining six T-cell epitopes (i.e., TCE-III, IV, V, VIII, IX, and X). Finally a combinatorial library was created containing beneficial diversity identified from multiple rounds of evolution that targeted TCE-I, III, IV, VIII, and X, along with C503 and C565, two amino acids reported to impact the aggregation state of PAL variants. The best hits from this library were subjected to further saturation mutagenesis targeting TCE-III and VIII and additional targeted mutagenesis at a few positions.

Construction and Screening of Deimmunizing Libraries:

[0261] Combinatorial and saturation mutagenesis libraries designed as described above were constructed by methods known in the art, and tested for activity in an unchallenged assay as described in Example 2. Active variants were identified and sequenced. Their activities and mutations with respect to AvPAL Variant No. 36 and numerous AvPAL Variants are provided in Tables 9-1 through 9-7, below.

Identification of Deimmunizing Diversity:

[0262] Active variants were analyzed for their levels of immunogenicity by evaluating their binding to the eight common Class II HLA-DR alleles described above. The total immunogenicity score and immunogenic hit count are shown in Tables 9-1 to 9-7. The total immunogenicity score reflects the overall predicted immunogenicity of the variant (i.e., a higher score indicates a higher level of predicted immunogenicity). The immunogenic "hit count" indicates the number of 15-mer analysis frames with an unusually high potential for immunogenicity (i.e., a higher hit count indicates a higher potential for immunogenicity). Mutations with a lower total predicted immunogenicity score and/or an immunogenic hit count less than that of the reference variant were considered to be "deimmunizing mutations." The deimmunizing mutations that were identified as being the best were recombined to generate a number of variants that are active and predicted to be significantly less immunogenic than the starting reference variant AvPAL. In the following Tables, the FIOP results are from the unchallenged assay; for the total immunogenicity score (TIS) and immunogenic hit count (IHC), the results are indicated for the whole PAL protein (Tables 9-1, 9-8, and 9-9) or for the indicated epitope (Tables 9-2 to 9-7).

Table 9-1. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-I, II, VI, and VII
("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)

Variant #	Active Mutations (as Compared to Variant No. 36)	FIOP	TIS	IHC
Variant 36		+	680	52
100	I27E/L214E	+	634	51
101	V105C/R134Q/Q205T/P266H/L278D	+	645	48
102	I27E/L214E/C503Q/A547D	+	629	51
103	V105C/Q205T/P210C/L214E/C503Q/A547D	+	619	49
104	I27E/A112C/R134Q/Q205T/I285E/C503Q	+	622	45
105	V39A/P266H	+	665	45
106	I27E/V39A/V105C/R134Q/P210C	+	607	41
107	I27E/V39A/V105C/P210C/L214E/P266H/L278D	+	590	42
108	V39A/V105C/L214E/P266H/A547D/C565N	+	613	42
109	I27E/V39A/A112C/L214E	+	616	42

Table 9-1. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-I, II, VI, and VII ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOP**	**TIS**	**IHC**
110	I27E/V39A/R134Q/A153G/L214E/P266H/I285E/C503Q/ A551D/ C565N	+	572	37
111	I27E/R134Q/L278D/I285E/A551D/C565N	+	626	48
112	I27E/V39A/V105C/A112C/Q205T/P266H/I285E/C503Q/A551D	+	594	39
113	I27E/V105C/L214E/P266H/C503Q	+	614	49
114	I27E/V39A/R134Q	+	637	44
115	C503Q/A547D	+	675	52
116	V105C/C503Q	+	660	50
117	I27E/R134Q/Q205T/P266H/L278D/A547D	+	641	50
118	I27E/V105C/R134Q/P210C/P266H/L278D/I285E/C503Q/A551D/C565N	+	596	45
119	I27E/V39A/V105C/R134Q/Q205T/L278D/I285E/C503Q/A547D/A551D/C565N	+	585	38
120	V105C/R134Q/L214E/C503Q/A547D/A551D	+	619	48
121	I27E/V39A/V105C/L214E/L278D/L309P/C503Q/A547D/A551D	+	587	42
122	V105C/L278D/C503Q/A551D	+	655	50
123	I27E/V105C/R134Q/P210C/L214E/P266H/L278D/A551D/C565N	++	591	48
124	I27E/R134Q/L214E/C503Q/A547D	++	620	50
125	R134Q/P210C/L214E/L278D/C503Q/A547D/C565N	+	630	50
126	I27E/V39A/V105C/Q205T/P210C/L214E/L278D/A547D	+	585	42
127	V39A/Q205T/L278D/A547D/A551D	+	654	44
128	V105C/R134Q/L214E/P266H/I285E/C503Q/A551D/C565N	+	598	45
129	I27E/V105C/L214E/A547D/A551D/C565N	+	609	49
130	I27E/V39A/V105C/Q205T/L278D/C503Q/A547D	+	615	42
131	I27E/V39A/V105C/R134Q/L214E/I285E/C503Q/A547D/A551D	+	564	38
132	C503Q/A547D/A551D/C565N	+	675	52
133	V105C/R134Q/L214E/C503Q/A547D	+	619	48
134	I27E/V105C/Q205T/C503Q/A547D/A551D/C565N	+	630	49
135	I27E/V39A	+	646	45
136	I27E/V39A/V105C/L214E/P266H/C503Q/A547D/C565N	+	594	42
137	A112C/R134Q/L214E/C503Q/A547D/A551D/C565N	+	636	48
138	I27E/A112C/R134Q/A153R/L214E/P266H/C503Q	+	619	47
139	C503Q/A551D	+	675	52
140	I27E/V39A/V105C/R134Q/L278D/C503Q/C565N	+	617	42
141	R134Q/Q205T/L214E/I285E/C503Q/A551D/C565N	+	613	47
142	V105C/L214E/I285E/A547D/C565N	+	607	46
143	I27E/V39A/R43L/L214E/A547D	+	625	45
144	I27E/V39A/P210C/L214E/I285E/C503Q/A551D	+	584	41
145	I27E/R134Q/L214E/C503Q/A547D/A551D	+	620	50
146	V39A/V105C/A153R/P266H/A547D/A551D	+	637	42
147	V39A/C503Q	+	665	45

Table 9-1. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-I, II, VI, and VII ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOP**	**TIS**	**IHC**
148	I27E/V39A/V105C/P210C/I285E/C503Q/A547D/A551D/C565N	+	590	39
149	R134Q/L214E/L278D/C503Q/A551D	+	639	50
150	I27E/V39A/R134Q/C503Q/A547D	+	632	44
151	A153R	+	677	51
152	I27E/V39A/V105C/A112C/R134Q/L214E/L278D/C503Q/A547D/C565N	+	585	41
153	C503Q/A547D/C565N	+	675	52
154	I27E/V39A/L214E/P266H/L278D/C503Q/A547D/A551D/C565N	+	614	44
155	L278D/A547D	+	675	52
156	V39A/G45S/L278D/C503Q/A551D	+	667	45
157	V39A/A153R/C503Q/A547D	+	657	44
158	R134Q/P210C/L214E/C503Q/A547D/A551D	+	630	50
159	I27E/A547D/C565N	+	656	52
160	V39A/R134Q/P210C/L214E/A547D/C565N	+	615	43
161	I27E/V39A/P210C/P266H/I285E/C503Q/A547D	+	610	41
162	I27E/V39A/R134Q/L278D/C503Q/A547D	+	632	44
163	I27E/V105C/R134Q/Q205T/P210C/C503Q	+	622	48
164	I27E/V105C/R134Q/L214E/L278D/C503Q/A547D	+	600	48
165	I27E/V105C/C503Q/A547D/C565N	+	636	50
166	I27E/L214E/C503Q	+	634	51
167	V105C/L214E/L278D/C503Q/A547D/A551D	+	628	49
168	I27E/V105C/Q205T/L214E/P266H	+	609	49
169	V39A/A112C/R134Q/L214E/C503Q/A547D/A551D	+	621	41
170	I27E/R134Q/L214E/C503Q/C565N	+	625	50
171	I27E/V39A/R134Q/A153R/Q205T/L214E/P266H/C503Q	+	602	42
172	I27E/V39A/L214E/C503Q/A551D	+	614	44
173	V39A/V105C/Q205T/A551D	+	634	42
174	I27E/V39A/Q205T/C503Q/A547D/C565N	+	635	44
175	A547D	+	675	52
176	I27E/V39A/V105C/R134Q/P210C/L214E/C503Q/A551D/C565N	+	576	41
177	V39A/P275R/L278D/C503Q/A551D	+	667	45
178	I27E/V39A/V105C/Q205T/P210C/L278D/A547D	+	611	42
179	V105C/A153R/Q205T/L214E/P266H/C503Q/A547D	+	620	48
180	V105C/A112C/R134Q/Q205T/L214E/Y492H/C503Q/A547D	+	614	48
181	I27E/P210C/L278D/C503Q	+	651	51
182	I27E/P210C/C503Q	+	651	51
183	I27E/V39A/R134Q/A153R/L214E/C503Q/A547D	+	602	42
184	I27E/P266H/A547D/A551D	+	656	52
185	V105C/L214E/I285E/C503Q/A547D/A551D/C565N	+	607	46
186	I27E/V105C/P266H/I285E/C503Q/A547D/C565N	+	615	47
187	Q205T/L278D/I285E/A547D/A551D	+	648	48

Table 9-1. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-I, II, VI, and VII ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOP**	**TIS**	**IHC**
188	V39A/V105C/Q205T/C503Q	+	639	42
189	I27E/V39A/Q205T/P266H/I285E/A547D/A551D/C565N	+	614	41
190	V105C/L214E/I285E	+	612	46
191	V105C/R134Q/C503Q/A547D/C565N	+	646	49
192	I27E/V39A/V105C/R134Q/C503Q/A551D	+	612	42
193	I27E/R134Q/Q205T/I285E/C503Q/A551D	+	620	47
194	I27E/V39A/A112C/Q205T/L278D/I285E	+	616	39
195	I27E/V39A/A112C/Q205T/L214E/P266H/C503Q/A551D/C565N	+	606	42
196	I27E/V39A/L278D/A547D	+	641	45
197	I27E/V39A/V105C/R134Q/Q205T/L214E/A551D/C565N	+	580	41
198	I27E/G45D/Q205T/P266H/C565N	+	656	51
199	I27E/V39A/A112C/L214E/L278D/C503Q/A547D/A551D	+	611	42
200	I27E/Q205T	+	655	51
201	I27E/A112C/R134Q/Q205T/L278D/C503Q	+	643	48
202	C565N	+	680	52
203	I27E/V39A/V105C/R134Q/Q205T/P210C/L278D/C503Q/A547D	+	602	41
204	V105C/L214E/P266H/L278D/A547D	+	628	49
205	V105C/R134Q/A153R/Q205T/L214E/C503Q	+	616	47
206	V105C/R134Q/C503Q	+	651	49
207	I27E/V39A/Q205T/L278D/C503Q/C565N	+	640	44
208	I27E/V39A/V105C/S131N/R134Q/Q205T/L214E/C503Q/A547D/C565N	+	579	41
209	Q205T/L214E/I285E/C503Q/A551D	+	622	48
210	I27E/L214E/L278D/C503Q	+	634	51
211	V105C/R134Q/L214E/L278D/C565N	+	624	48
212	I27E/V39A/L214E	+	619	44
213	L214E/C503Q/A547D	+	648	51
214	L214E/P266H	+	653	51
215	I27E/V39A/C503Q	+	646	45
216	P210C/L214E	+	644	51
217	V105C	+	660	50
218	A112C/R134Q/A153R/L214E/L278D/I285E/C503Q/A547D/A551D/C565N	+	612	44
219	I27E/Q205T/L278D/A551D	+	650	51
220	I27E/L214E/A551D	+	629	51
221	R134Q/L214E/I285E/C503Q	++	623	47
222	I27E/V39A/V105C/C503Q/A551D	+	621	43
223	A112C/L278D/C503Q/A547D	+	672	50
224	I27E/R134Q/A153R/I285E/C503Q/A547D	++	623	47
225	I27E/V39A/V105C/A153R/I285E	+	602	39
226	I27E/V39A/R134Q/L278D/I285E/C503Q/A547D/A551D	++	611	41

Table 9-1. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-I, II, VI, and VII ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOP**	**TIS**	**IHC**
227	R134Q/C503Q	+	671	51
228	V39A/C503Q/A551D/C565N	+	660	45
229	I27E/V39A/P266H	+	646	45
230	V105C/I285E	+	639	47
231	I27E/V39A/V105C/A153R/L214E	+	596	41
232	A112C/L214E/L278D	+	650	49
233	I27E/R134Q/P210C	+	642	50
234	I27E/A153R/L214E/L278D/A551D	+	626	50
235	V105C/I285E/A547D	+	634	47
236	I27E/Q205T/L214E/L278D/I285E/C503Q/C565N	+	608	48
237	I27E/V39A/P210C/T212S	+	636	44
238	I27E/P266H/L278D/C503Q	+	661	52
239	I27E/V105C/Q205T/L214E/P266H/A551D/C565N	+	604	49
240	I27E/A112C/P210C/L214E/C503Q/A547D	+	617	49
241	I27E/V39A/R134Q/P210C/C503Q/A551D	+	622	43
242	I27E/V39A/V105C/I285E/A547D	+	600	40
243	I27E/V39A/Q205T/L278D/C503Q/A551D/C565N	+	635	44
244	I27E/R134Q/I285E/C503Q	+	631	48
245	I27E/V39A/P210C/L214E/L278D/C503Q/A551D	+	605	44
246	V39A/A112C/R134Q/Q205T/L214E/L278D	+	621	41
247	I27E/V39A/L214E/L278D/C503Q	+	619	44
248	I27E/V105C/L214E	+	614	49
249	I27E/V39A/V105C/R134Q/P266H/C503Q/A547D/A551D	+	612	42
250	I27E/V39A/P210C/P266H/C503Q/A551D	+	631	44
251	I27E/V39A/V105C/A153R/C503Q/A547D/C565N	+	618	42
252	V39A/V105C/R134Q/L214E/C503Q/A547D/A551D	+	604	41
253	I27E/L214E/L278D	+	634	51
254	I27E/V39A/V105C/R134Q/A153R/P210C/L278D/I285E/C503Q/A547D/A551D	+	578	37
255	V105C/R134Q/Q205T/L214E/A547D	+	614	48
256	I27E/V105C/R134Q/A153R/C503Q	+	629	48
257	V105C/R134Q/C503Q/A547D	+	646	49
258	I27E/V39A/L278D/I285E/C503Q/A547D	+	620	42
259	I27E/V39A/R134Q/A153R/L278D	+	634	43
260	I27E/V39A/A112C/R134Q/L214E/C503Q/A547D	+	602	41
261	I27E/V39A/R134Q/A153R/L278D/A547D/A551D	+	629	43
262	I27E/V39A/L278D/C503Q/C565N	+	646	45
263	Q205T/L214E/C503Q/A547D/C565N	+	643	51
264	R134Q/L214E	+	644	50
265	I27E/V39A/P266H/L278D	+	646	45
266	R134Q/Q205T/C503Q	+	665	50

Table 9-1. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-I, II, VI, and VII ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOP**	**TIS**	**IHC**
267	V39A/V105C/R134Q/P210C/L214E/A551D	+	595	41
268	V105C/A153R/Q205T/P266H/I285E/A547D/C565N	+	625	45
269	I27E/V39A/R134Q/L214E/P266H/A551D	+	605	43
270	L214E/P266H/C503Q/A547D/A551D/C565N	+	648	51
271	V39A/V105C/P210C/A547D	+	630	42
272	I27E/V105C	+	641	50
273	I27E/V39A/R134Q/Q205T/L214E/P266H/I285E/C503Q/A551D/C565N	+	579	40
274	I27E/V39A/R134Q/Q205T/P266H/C503Q/A551D/C565N	+	626	43
275	I27E/V105C/R134Q/P210C/L214E/C503Q/A551D/C565N	+	591	48
276	I27E/L214E/C503Q/A547D/C565N	+	629	51
277	I27E/V39A/R134Q/A153R/P210C/L214E/L278D/I285E/A547D/C565N	+	572	39
278	I27E/V39A/A112C/I285E	+	622	40
279	I27E/A112C/R134Q/L278D/I285E/C503Q/A551D/C565N	+	623	46
280	I27E/L214E/P266H/L278D/I285E/A551D	+	608	48
281	I27E/V39A/V105C/A153R/A551D/C565N	+	618	42
282	V39A/V105C/R134Q/A153R/Q205T/A551D	+	622	40
283	I27E/V39A/V105C/R134Q/Q205T/L214E	+	585	41
284	I27E/V105C/Q205T	+	635	49
285	I27E/Q205T/P266H/L278D/I285E/A551D/C565N	+	629	48
286	I27E/R134Q/L214E/C503Q	+	625	50
287	V105C/Q205T/L214E/C503Q/A547D/A551D/C565N	+	623	49
288	V39A/V105C/L214E/I285E/C503Q/A551D/C565N	+	592	39
289	I27E/V39A/V105C/L278D/C503Q/A547D/C565N	+	621	43
290	I27E/V39A/V105C/R134Q/I285E/A547D/A551D	+	591	39
291	I27E/V39A/A112C/R134Q/L214E/P266H/A551D	+	602	41
292	I27E/L278D/C503Q/A551D	+	656	52
293	I27E/V39A/R134Q/A153R/A547D	+	629	43
294	P266H/C565N	+	680	52
295	I27E/V105C/I285E/C503Q/A547D/A551D/C565N	+	615	47
296	I27E/V39A/L278D	+	646	45
297	P210C/L214E/P266H	+	644	51
298	I27E/V39A/R134Q/P210C/L214E/C503Q	+	601	43
299	I27E/V39A/R43L/V105C/A153R/L214E/P266H/L278D/C503Q	+	607	42
300	I27E/V105C/Q205T/L214E/L278D/I285E/C503Q/A547D/A551D/C565N	+	583	46
301	I27E/V39A/V105C/C503Q/A547D/A551D/C565N	+	621	43
302	I27E/V105C/L214E/I285E/A551D/C565N	+	588	46
303	I27E/P210C/A551D	+	646	51
304	I27E/V39A/V105C/Q205T/L214E/L278D/C503Q/A547D	+	589	42

Table 9-1. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-I, II, VI, and VII ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOP**	**TIS**	**IHC**
305	I27E/V105C/L278D/A547D	+	636	50
306	C503Q	+	680	52
307	I27E/V39A/V105C/R134Q/L214E/L278D/A547D/A551D	+	585	41
308	I27E/R134Q	+	652	51
309	V39A/R134Q	+	656	44
310	I27E/V39A/V105C/A112C/Q205T/P210C/P266H/C503Q/A547D	+	611	42
311	V39A/A112C/A153R/Q205T/L278D/C503Q/A547D	+	648	41
312	I27E/V105C/P266H	+	641	50
313	I27E/V39A/V105C/R134Q/P210C/L214E	+	581	41
314	I27E/V39A/V105C/R134Q/L278D/A551D	+	612	42
315	I27E/V39A/V105C/A112C/R134Q/A153R/Q205T/L214E/P266H/L278D/C503Q/A551D	+	577	40
316	I27E/V105C/R134Q/A153R/I285E/A547D	+	603	45
317	I27E/V39A/A112C/A547D	+	638	43
318	I27E/V39A/R134Q/A153R/L214E/P266H/L278D/C503Q/A547D/C565N	+	602	42
319	I27E/V105C/Q205T/P266H/C503Q	+	635	49
320	I27E/V39A/V105C/R134Q/L278D/I285E/C503Q	+	596	39
321	C503Q/A551D/C565N	+	675	52
322	I27E/V39A/A112C/R134Q/Q205T/P210C/L214E/A551D/C565N	+	593	41
323	R134Q/C503Q/A547D/A551D	+	666	51
324	I27E/A112C/Q205T/P266H/L278D/I285E/C503Q	+	631	46
325	P266H/L278D/C503Q	+	680	52
326	I27E/V39A/Q205T/P266H/A551D	+	635	44
327	Q205T/L214E/I285E/C503Q/C565N	+	627	48
328	I27E/V105C/R134Q/Q205T/L214E/P266H/L278D/C503Q/C565N	+	600	48
329	I27E/V39A/V105C/R134Q/P266H/C503Q	+	617	42
330	I27E/V39A/V105C/R134Q/P210C/L214E/I285E/A547D	+	555	38
331	I27E/V39A/V105C/Q205T/P210C/L278D/C503Q	+	616	42
332	I27E/V39A/R134Q/A547D/C565N	+	632	44
333	V105C/A547D/A551D	+	655	50
334	I27E/V105C/R134Q/A153R/P210C/L214E/C503Q/A547D	+	588	47
335	I27E/P210C/L214E/C503Q/A547D	++	620	51
336	I27E/V105C/C503Q/A547D/A551D/C565N	+	636	50
337	I27E/V105C/R134Q/L214E/L278D/C503Q/A547D/A551D/C565N	+	600	48
338	I27E/V39A/V105C/A153R/Q205T/L278D/C503Q/A547D/A551D	+	612	41
339	I27E/V39A/V105C/A112C/L214E/I285E/C503Q/A547D	+	573	39
340	L214E/A547D	+	648	51
341	V105C/Q205T/L214E/L278D	+	628	49
342	I27E/V39A/Q205T/L214E/C503Q/C565N	+	614	44
343	I27E/P210C	+	651	51

Table 9-1. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-I, II, VI, and VII ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOP**	**TIS**	**IHC**
344	V105C/Q205T/C503Q/A551D	+	649	49
345	I27E/V39A/A112C/R134Q/P266H/I285E	+	613	39
346	V39A/P210C/L214E/L278D/I285E/C503Q/A551D	+	603	41
347	I27E/Q205T/L214E/C503Q/A547D/C565N	+	624	51
348	I27E/V39A/L214E/L278D/C503Q/A547D/C565N	+	614	44
349	I27E/P210C/C503Q/C565N	+	651	51
350	I27E/L278D/C503Q/A551D/C565N	+	656	52
351	R134Q/L214E/L278D/C503Q	+	644	50
352	I27E/A153R/L214E/L278D/I285E/A551D/C565N	+	605	47
353	I27E/V105C/L214E/A551D/C565N	+	609	49
354	V39A/R134Q/Q205T/L214E/C503Q/C565N	+	624	43

Table 9-2. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-III ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOPC**	**TIS**	**IHC**
Variant 36		+	71	7
400	V80I/R134C/P564Q	+	60	5
401	V121C	+	44	6
402	R134L	+	78	7
403	A124G	+	58	7
404	R134W	+	63	5
405	L126M	+	56	5
406	N130C/M370I	+	62	6
407	N130Q	+	65	7
408	A123G	+	67	8
409	A129G	+	64	9
410	G135A/A394E	+	76	7
411	A129L	+	82	9
412	M133R	+	52	3
413	G135S	+	78	9
414	L126T	+	62	8
415	L127A	+	46	6
416	R134I	+	82	7
417	R134N/G307C	+	60	5
418	L126I	++	80	12
419	G135C	+	67	6
420	M125L	+	72	8

Table 9-3. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-1V ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOPC**	**TIS**	**IHC**
Variant 36		+	74	11
500	P157H	+	69	8
501	R140M	+	72	11
502	P157F	+	80	12
503	A153C	+	56	7
504	E142V	+	84	14
505	K145G/P157T	+	62	8
506	R140D	+	63	8
507	E142D/G371D	+	79	13
508	M147A	+	48	1
509	T156K/G483C	+	83	12
510	P157D	+	64	8
511	A62S/M147V	+	62	8
512	R146L	+	81	14
513	Y158E	+	72	11
514	G154Y/L174M/Q321K/S456I/G483C	+	75	10
515	S82I/G135C/P157F/W279L	+	77	11
516	I144N	+	61	7
517	T110I/I139R	+	65	8
518	L47M/I144L	+	67	8
519	L47M/M147G/A383E	+	48	4
520	G20S/I144L	+	67	8
521	R146W/D191Y	+	62	5
522	P157Y	+	73	9
523	L47M/P157C	+	68	8
524	E142P	+	68	9
525	F150K	+	59	6
526	L141T	+	55	7
527	V159H	+	67	8
528	I144L	+	67	8
529	A119E/T156H/A289D	+	78	11
530	Q58K/P157D/G369C	+	64	8
531	L47M/R146E	+	58	6
532	E142H	+	79	13
533	R140N/A199E	+	67	8
534	I144V	+	72	13
535	I149L	+	69	9
536	R146H	+	77	12
537	I139M	+	74	11
538	A153S/H250N	+	73	10
539	L319M	+	74	11
540	R140E/A334S/A551D	+	63	8
541	F150L	+	79	12
542	L143M	+	72	11
543	A153G	+	62	8

Table 9-3. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-1V ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOPC**	**TIS**	**IHC**
544	I139V	+	74	11
545	Q58H/L143V	+	72	11
546	G154R	+	81	13
547	K145Q	+	72	11
548	L143F	+	74	11
549	R140G	+	67	8
550	V159C	+	72	11
551	Q389K	+	74	11
552	L141P	+	58	5
553	M247I	+	74	11
554	F150M	+	77	12
555	L141Q	+	59	7
556	L151M	+	68	9
557	V159L	+	74	11
558	R94C/I149E	+	53	6
559	V159M	+	74	11
560	L118M/L141H	+	58	7
561	K145N	+	71	11
562	I149R	+	72	11
563	K145R	+	84	15
564	L141K	+	59	7
565	R43S	+	74	11

Table 9-4. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-V. ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant No.	**Active Mutations (as Compared to Variant No. 36)**	**FIOPC**	**TIS**	**IHC**
Variant 36		+	36	2
600	Y176R	+	51	4
601	S180C	+	27	0
602	S180T	+	27	0
603	V172L	+	30	1
604	I177V	+	24	0
605	V172I	+	29	1
606	I177M	+	30	1
607	Y176I	+	54	4
608	Y176M	+	33	2
609	Y176V	+	41	3
610	L174M	+	21	0
611	P117T/Y176Q	+	37	2

Table 9-4. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-V. ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant No.	**Active Mutations (as Compared to Variant No. 36)**	**FIOPC**	**TIS**	**IHC**
612	S175G	+	26	0
613	T178L/A477S	+	43	5
614	V172C	+	26	0
615	Y176E	+	20	0

Table 9-5. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-VIII. ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOPC**	**TIS**	**IHC**
Variant 36		+	83	10
700	A232S	+	83	10
701	A112S/M370A/A507E	+	71	10
702	Q240K/H374R	+	83	10
703	S461G	+	83	10
704	H374D	+	83	10
705	M372A	+	74	10
706	L349M	+	83	10
707	Y378S	+	74	7
708	G371H	+	91	11
709	Y377N	+	82	10
710	I379N	+	80	8
711	D191Y/H385N	+	81	10
712	I379L	+	83	10
713	R43S/H374R	+	83	10
714	Q355K/H374S	+	83	10
715	P275T/H374R	+	83	10
716	H374Q/P396Q	+	99	14
717	H385N	+	81	10
718	Y378L	+	78	9
719	I379C	+	70	7
720	M370G	+	64	10
721	M372V	+	76	10
722	K384R	+	87	10
723	A383V	+	88	13
724	M147I/H374S	+	83	10
725	Y378F/P404Q	+	85	10
726	H374S	+	83	10
727	Y378E	+	55	2
728	H374R/G417C	+	83	10
729	L418M	+	83	10
730	S525L	+	83	10
731	Y378D	+	60	4

Table 9-5. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-VIII. ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOPC**	**TIS**	**IHC**
732	A383S	+	88	10
733	D387S	+	96	11
734	L382H	+	78	10
735	L382C	+	76	8
736	G371Q	+	84	10
737	H374L	+	85	11
738	Y378C	+	58	5
739	H374A	+	83	10
740	L375M	+	77	10
741	H385C	+	74	8
742	A334S/H374V	+	85	11
743	H374R	+	83	10
744	H385M/P403H	+	83	11
745	Y378I	+	83	10
746	Y377I	+	86	10
747	H385G	+	74	7
748	H385S/P403H	+	79	9
749	H374N	+	83	10
750	S187R/L381V	+	78	7
751	L382S	+	77	9
752	Y377C	+	75	7
753	L381V	+	78	7
754	G371S	+	88	10
755	A256S/L381N	+	70	6
756	I379M	+	80	10
757	R43S/H374K	+	83	10
758	I379H	+	76	7
759	M370S	+	64	10
760	P275Q/M370S	+	64	10
761	G425V	+	83	10
762	A447S	+	83	10
763	L382M	+	80	10
764	G371N	+	84	10
765	L381M/Q560K	+	79	10
767	L382I	+	77	8
768	H374G	+	83	10
769	M370I	+	76	10
770	Q332K/Y377M	+	86	10
771	Y378N	+	64	5
772	L375I	+	83	10
773	H374T	+	83	10
774	L381G	+	60	4

Table 9-6. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-IX. ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant No.	**Active Mutations (as Compared to Variant No. 36)**	**FIOP C**	**TIS**	**IHC**
Variant 36		+	45	4
800	I439V	+	49	4
801	I439C	+	39	1
802	S438F	+	50	4
803	T433S	+	44	0
804	T433A	+	35	0
805	Y435Q/H446N	+	43	2
806	L431V	+	28	0
807	N437Q	+	40	0
808	L213M/S438L	+	46	4
809	L432C	+	39	2
810	L432V	+	47	4
811	T433L	+	50	4
812	L431P	+	18	0
813	I439F	+	47	4
814	S286R/Y435T	+	53	4
815	S98I	+	45	4
816	S438R	+	48	4
817	S331I	+	45	4
818	S438M	+	44	4
819	Q240K/T433Y	+	41	0
820	T433I	+	46	3
821	T433N	+	38	0
822	L431S	+	27	0
823	N437G	+	38	0
824	L431E	+	26	0
825	L431C	+	25	0
826	G436T	+	45	0
827	G436D	+	42	1
828	T433V	+	59	7
829	N437T/L538M	+	52	4
830	A289S/L431E	+	26	0
831	T433Q	+	37	0
832	I439L	+	46	4
833	F434C	+	34	0
834	T433P	+	35	0
835	A24S/F434M	+	69	8
836	L431G	+	18	0
837	S438C	+	45	1
838	T433W	+	33	0
839	Y435L	+	57	5
840	A62S/T433N	+	38	0
841	N437E	+	46	3
842	A477S	+	45	4
843	G436M	+	87	11
844	T433R	+	70	10
845	S438T	+	46	4

Table 9-7. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-X. ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOPC**	**TIS**	**IHC**
Variant 36		+	47	7
900	I471V	+	45	7
901	F472G	+	16	0
902	G483S	+	60	9
903	G483A/S524I	+	64	10
904	Y475C	+	20	0
905	I478N	+	44	6
906	V476I	+	30	2
907	N474D/R490H	+	29	3
908	P275Q	+	47	7
909	Q473K	+	55	7
910	G483H	+	58	8
911	Q473H/A507S	+	52	7
912	N474H	+	49	7
913	Y475F	+	58	7
914	I478S	+	46	6
915	Q473M	+	69	9
916	V476C	+	23	0
917	L104M/V476L	+	36	3
918	A119E/G365A	+	47	7
919	N474W	+	51	7
920	I471K	+	29	2
921	Q292H/A479G	+	35	2
922	G276V	+	47	7
923	I471N	+	26	1
924	A479S	+	53	7
925	A558S	+	47	7
926	Q473S	+	56	8
927	Q473R	+	62	8
928	Y475Q	+	33	5
929	I471G	+	32	3
930	A479G	+	35	2
931	A70S/N474E	+	32	3
932	I471F	+	48	7
933	Q58R/Y475H	+	32	5
934	F482L	+	56	9
935	V476L	+	36	3
936	A24E	+	47	7
937	N474R	+	55	7
938	G483C	+	49	6
939	Q473H	+	52	7
940	I471M	+	46	7
941	I471R	+	31	2
942	P404T/A477V	+	47	6
943	G483R/G537C	+	78	13

Table 9-7. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-X. ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOPC**	**TIS**	**IHC**
944	F482C	+	45	6
945	Q355H/I478C	+	31	1
946	L206M	+	47	7
947	N474A	+	48	7
948	Y475L	+	60	7
949	I471W	+	34	4

Table 9-8. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-I, III, IV, VIII, and X ("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 36)**	**FIOP**	**TIS**	**IHC**
Variant 36		+		
950	F472G/C503Q/C565N	+	651	45
951	L381G/F472GC503Q/C565N	+	628	39
952	M133R/C503Q	+	663	48
953	M133R/L381G/C565N	+	640	42
954	V39A/C503Q/C565N	+	667	45
955	V39A/F472G/C503Q/C565N	+	636	38
956	V39A/G248C/L381G/F472G/C503Q/C565N	+	613	32
957	V39A/K115E/M133R/C565N	++	646	39
958	V39A/L381G	+	644	39
959	V39A/M133R/C503Q	+	648	41
960	V39A/M133R/C503Q/C565N	+	648	41
961	V39A/M133R/F472G/C503Q/C565N	+	617	34
962	V39A/M133R/F472G/C565N	+	617	34
963	V39A/M147A/C565N	+	641	35
964	V39A/M147A/F472G/C503Q/C565N	+	610	28
965	V39A/M147A/F472G/C565N	+	610	28
966	V39A/M147A/L381G/C503Q/C565N	+	618	29
967	V39A/M147A/L381G/F472G/C503Q/C565N	+	587	22
968	V39A/M147A/Y378E/C503Q/C565N	+	613	27
969	V39A/M147A/Y378E/C565N	+	613	27
970	V39A/Y378E/C503Q/C565N	+	639	37
971	V39A/Y378E/C565N	+	639	37
972	Y378D/C503Q	+	659	46
973	Y378E/F472G/C503Q/C565N	+	623	37

Table 9-9. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-III and VIII				
("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 967)**	**FIOP**	**TIS**	**IHC**
Variant 967		+		
974	A129I	-	598	26
975	A129V	-	595	24
976	A136K	++	595	22
977	A24E/G381L	+	604	28
978	A289S	+	590	23
979	A383C	+	577	18
980	A383M	++	602	28
981	G381A	++	598	24
982	G381C	-	580	22
983	G381F	+	601	25
984	G381I	++	610	27
985	G381L	++	610	28
986	G381M	+	606	28
987	G381N	+	597	24
988	G381Q	+	590	23
989	G381S	+	601	24
990	G381T	+	590	23
991	H132L	-	603	25
992	H132S	-	592	23
993	H374G	+	587	22
994	H374M	+	589	23
995	H374Q	-	587	22
996	L127V	-	576	22
997	L431M	+	591	22
998	L563M	-	587	22
999	M372L	-	580	22
1000	R134C	-	576	20
1001	R134F	+	588	21
1002	R134H	+	579	21
1003	R134K	-	585	21
1004	S131C	-	579	21
1005	S131T	+	580	21
1006	V388C	+	577	21

Table 9-9. FIOP, Total Immunogenicity (TIS), and Immunogenic Hit Count (IHC) for Variants Targeting TCE-III and VIII				
("-" = FIOP < 0.7; "+"= FIOP 0.7<FIOP<1.4; "++" = FIOP> 1.4)				
Variant #	**Active Mutations (as Compared to Variant No. 967)**	**FIOP**	**TIS**	**IHC**
1007	V388T	+	578	21
1008	R134H/V388T	+	570	20
1009	R134H/Y378E/G381L	+	574	19
1010	R134H/Y378E/G381L/V388T	+	566	19

***In vitro* Testing of Deimmunized PAL Variants:**

[0263] The deimmunized PAL variants are tested in a dendritic T-cell assay to empirically test their capacity to elicit a T-cell response. Peripheral blood mononuclear cells (PBMCs) are isolated from a human donor using standard techniques. These cells are used as a source of monocytes that are cultured in defined media to generate immature dendritic cells. These immature dendritic cells (DCs) are loaded with deimmunized PAL variants, and are then induced into a more mature phenotype by further culturing in defined media to provide antigen-primed DCs. CD8+ T cell-depleted donor PBMCs obtained from the same donor samples as the DCs are labeled with CFSE, then cultured with the antigen-primed DCs for 7 days, after which octuplicates are tested. Each DC-T cell culture includes a set of untreated controls (i.e., negative controls). The assay also incorporates reference antigen controls (i.e., positive controls), comprising two potent whole protein antigens. Assays utilizing cells isolated from 50 human donors with diverse Major Histocompatibility Complex Class II alleles provide a statistically relevant assessment of the PAL variants' capacity to elicit a T-cell response.

[0264] While the invention has been described with reference to the specific embodiments, various changes can be made and equivalents can be substituted to adapt to a particular situation, material, composition of matter, process, process step or steps, thereby achieving benefits of the invention without departing from the scope of what is claimed.

[0265] For all purposes in the United States of America, each and every publication and patent document cited in this disclosure is incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference. Citation of publications and patent documents is not intended as an indication that any such document is pertinent prior art, nor does it constitute an admission as to its contents or date.

The Claims defining the invention are as follows:

1. An engineered polypeptide comprising: a) an amino acid sequence having at least 90% sequence identity to reference sequence SEQ ID NO:4, where said amino acid sequence comprises an amino acid residue difference at position H307G/Q/M; and wherein said engineered polypeptide further comprises at least one or more of the following substitutions A39V, T54K, G59R, S73K, A112C, R134Q, A91V, Y158H, S180A, K195E, Q240R/W, T243I/L, I245L, A256G, L257W/A, N270K, N290G, Y304H, R305M, E308Q, I326F, L349M, D353A/N, L364Q, A394V, S399N, N400K, P404A, L407V, F443H, N453G, Y459F, T460G, T463N, N474Q, E509L, Q521K/S, K522Y/F/N, T524S, P528L, S546R, and P564G/L/M, when optimally aligned with the polypeptide of SEQ ID NO:4, and wherein said engineered polypeptide b) exhibits an improved property selected from i) reduced sensitivity to proteolysis, ii) increased tolerance to acidic pH, iii) reduced immunogenicity, or a combination of any of i), ii), or iii), as compared to the reference sequence SEQ ID NO:4.

2. The engineered polypeptide of Claim 1, wherein the improved property is selected from reduced sensitivity to proteolysis and/or increased tolerance to acidic pH.

3. The engineered polypeptide of Claim 1, wherein the engineered polypeptide has at least 90% sequence identity to reference SEQ ID NO:4.

4. The engineered polypeptide of Claim 1, wherein said engineered polypeptide is resistant to proteolysis, acid stable, and/or deimmunized.

5. The engineered polypeptide of Claim 4, wherein said engineered polypeptide is resistant to proteolysis by at least one digestive tract enzyme, wherein said engineered polypeptide is resistant to proteolysis by chymotrypsin, trypsin, carboxypeptidases, and/or elastases.

6. The engineered polypeptide of Claim 1, wherein said engineered polypeptide is deimmunized.

7. The deimmunized engineered polypeptide of Claim 6, wherein said polypeptide comprises an amino acid sequence having at least 90% sequence identity to SEQ ID NO:8.

8. The engineered polypeptide of Claim 1, wherein said polypeptide is purified.

9. A polynucleotide sequence encoding at least one engineered polypeptide of Claim 1.

10. The polynucleotide sequence of Claim 9, wherein said polynucleotide sequence is operably linked to a control sequence.

11. The polynucleotide sequence of Claim 9, wherein said polynucleotide sequence is codon-optimized.

12. An expression vector comprising at least one polynucleotide sequence of Claim 9 and at least one control sequence.

13. The expression vector of Claim 12, wherein said control sequence is a promoter.

14. A host cell transformed with at least one expression vector of Claim 12.

15. A method of producing an engineered polypeptide in a host cell comprising culturing a host cell comprising at least one expression vector of Claim 12, under suitable culture conditions, such that at least one engineered PAL polypeptide is produced.

16. The method of Claim 15, further comprising recovering at least one engineered polypeptide from the culture and/or host cells.

17. The method of Claim 16, further comprising the step of purifying said at least one engineered polypeptide.

18. A composition comprising at least one engineered polypeptide of Claim 1.

19. The composition of Claim 18, wherein said composition is a pharmaceutical composition.

20. The composition of Claim 19, wherein said composition is suitable for the treatment of phenylketonuria.

21. The pharmaceutical composition of Claim 20, wherein said composition is suitable for oral administration to a human.

22. The pharmaceutical composition of Claim 21, wherein said composition is in the form of a pill,

tablet, capsule, gelcap, liquid, or emulsion.

23. The pharmaceutical composition of Claim 22, wherein said pill, tablet, capsule, or gelcap further comprises an enteric coating.

24. The pharmaceutical composition of Claim 20, wherein said composition is suitable for parenteral injection into a human.

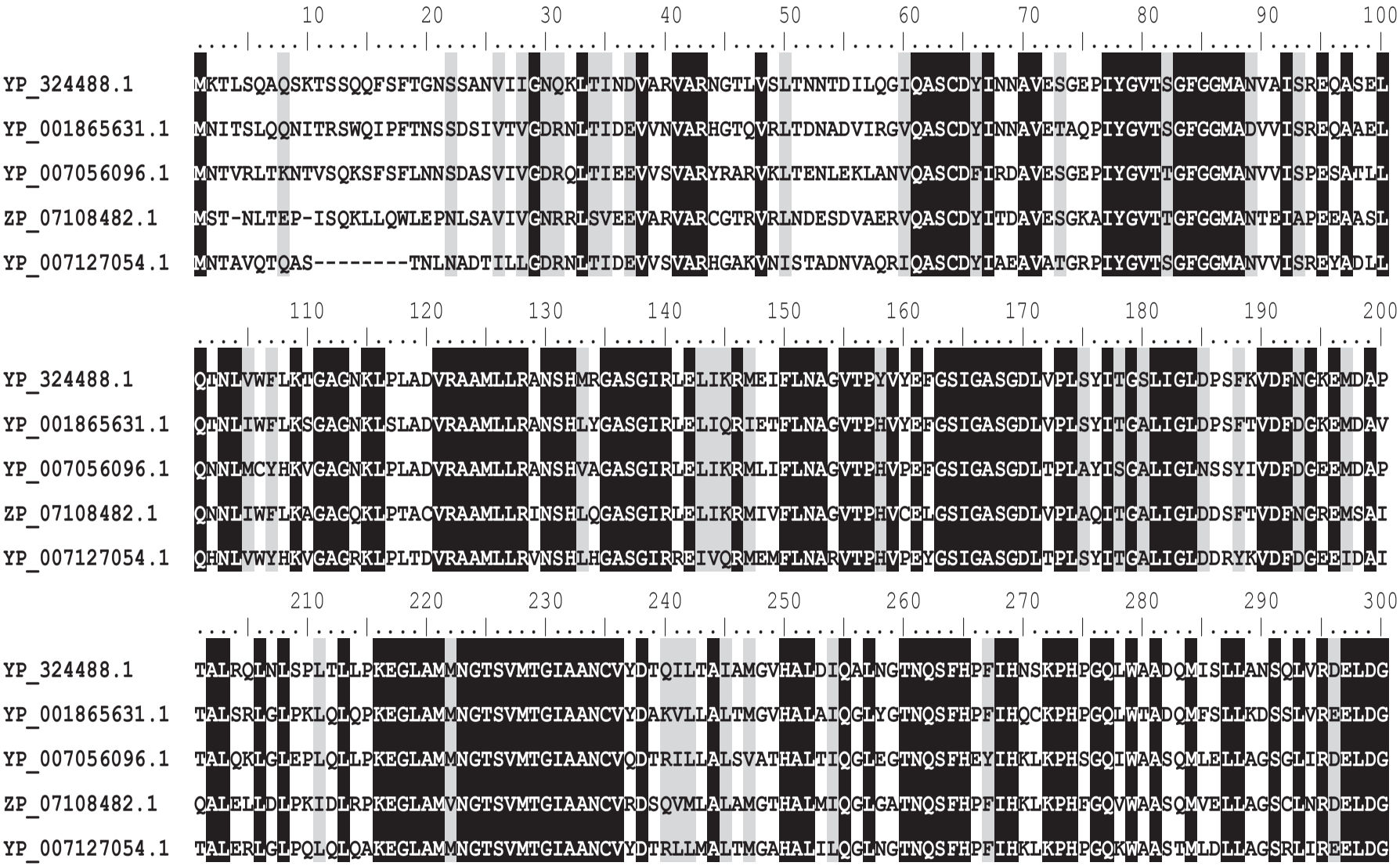

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ZP_07108482.1 MST-NLTEP-ISQKLLQWLEPNLSAVIVGNRRLSVEEVARVARCGTRVRLNDESDVAERVQASCDYITDAVESGKAIYGVTTGFGGMANTEIAPEEAASL
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110 120 130 140 150 160 170 180 190 200
YP_324488.1 QTNLVWFLKTGAGNKLPLADVRAAMLLRANSHMRGASGIRLELIKRMEIFLNAGVTPYVYEFGSIGASGDLVPLSYITGSLIGLDPSFKVDFNGKEMDAP
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YP_007127054.1 QHNLVWYHKVGAGRKLPLTDVRAAMLLRVNSHLHGASGIRREIVQRMEMFLNARVTPHVPEYGSIGASGDLTPLSYITGALIGLDDRYKVDFDGEEIDAI
210 220 230 240 250 260 270 280 290 300
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FIG. 1

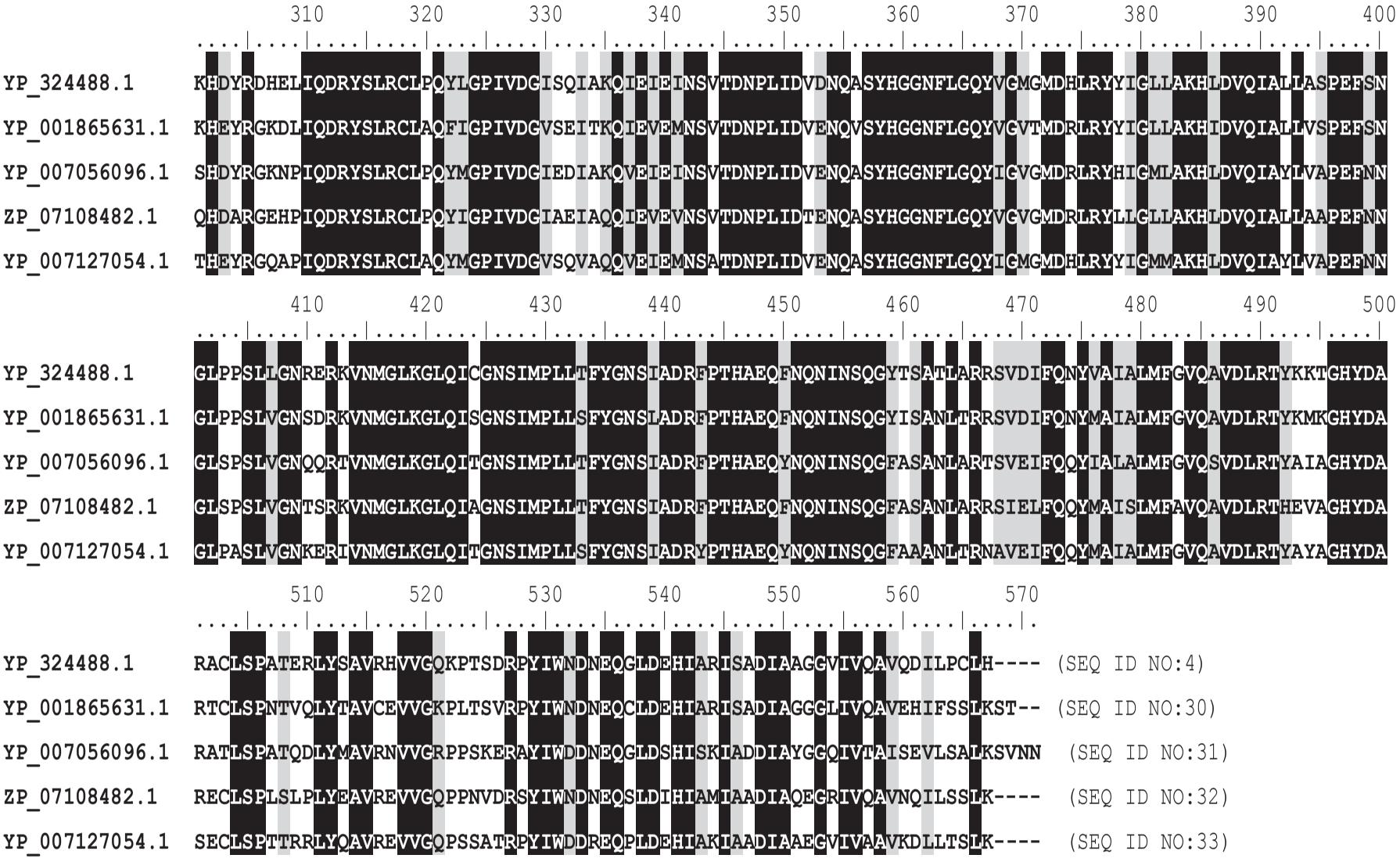

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ZP_07108482.1 QHDARGEHPIQDRYSLRCLPQYIGPIVDGIAEIAQQIEVEVNSVTDNPLIDTENQASYHGGNFLGQYVGVGMDRLRYLLGLLAKHLDVQIALLAAPEFNN
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410 420 430 440 450 460 470 480 490 500
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YP_007056096.1 RATLSPATQDLYMAVRNVVGRPPSKERAYIWDDNEQGLDSHISKIADDIAYGGQIVTAISEVLSALKSVNN (SEQ ID NO:31)
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YP_007127054.1 SECLSPTTRRLYQAVREVVGQPSSATRPYIWDDREQPLDEHIAKIAADIAAEGVIVAAVKDLLTSLK---- (SEQ ID NO:33)

FIG. 1 (Cont.)

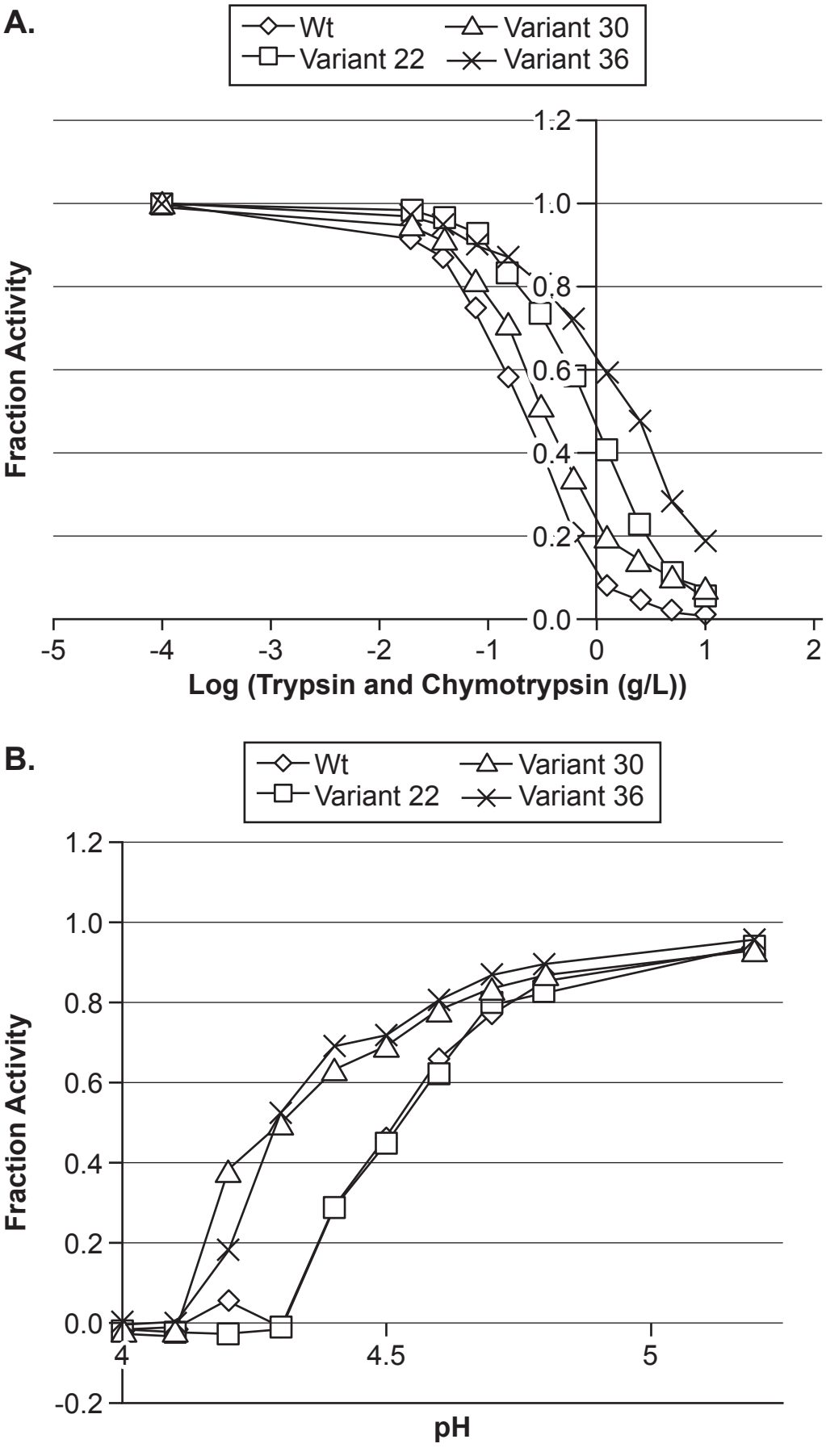

A.
Wt
Variant 30
Variant 22
Variant 36
Fraction Activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-5
-4
-3
-2
-1
0
1
2
Log (Trypsin and Chymotrypsin (g/L))
B.
Wt
Variant 30
Variant 22
Variant 36
Fraction Activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
4
4.5
5
pH

FIG. 2

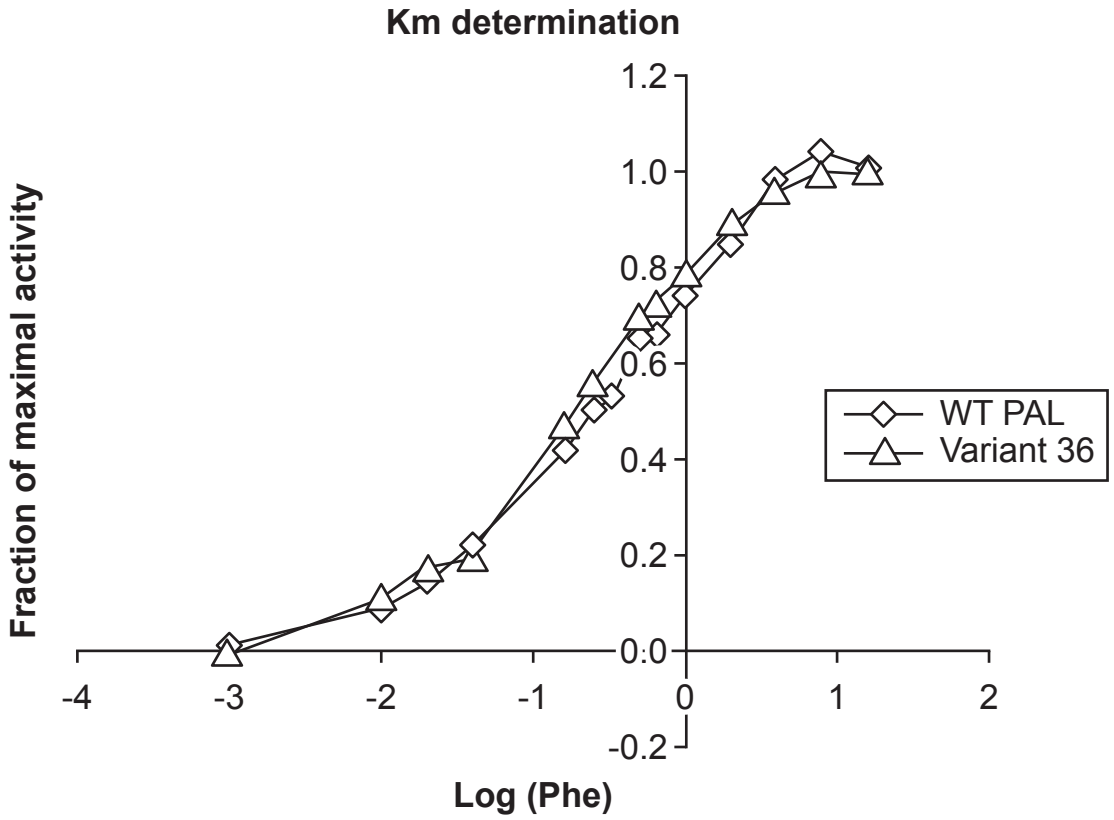

Km determination
Fraction of maximal activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-4
-3
-2
-1
0
1
2
Log (Phe)
WT PAL
Variant 36

FIG. 3

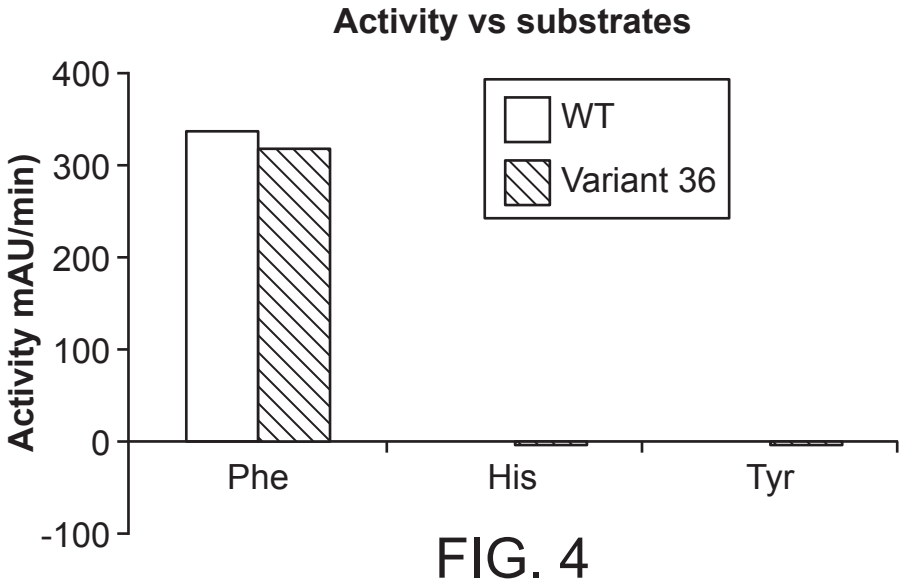

Activity vs substrates
Activity mAU/min)
400
300
200
100
0
-100
Phe
His
Tyr
WT
Variant 36

FIG. 4

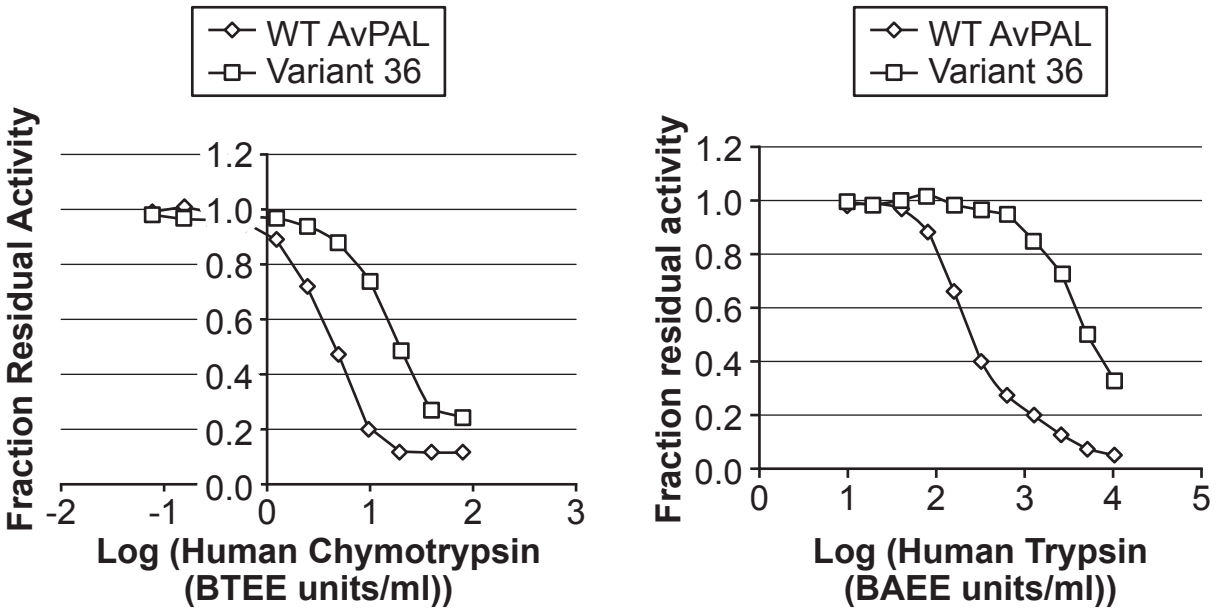

WT AvPAL
Variant 36
Fraction Residual Activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-2
-1
0
1
2
3
Log (Human Chymotrypsin (BTEE units/ml))
WT AvPAL
Variant 36
Fraction residual activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Log (Human Trypsin (BAEE units/ml))

FIG. 5

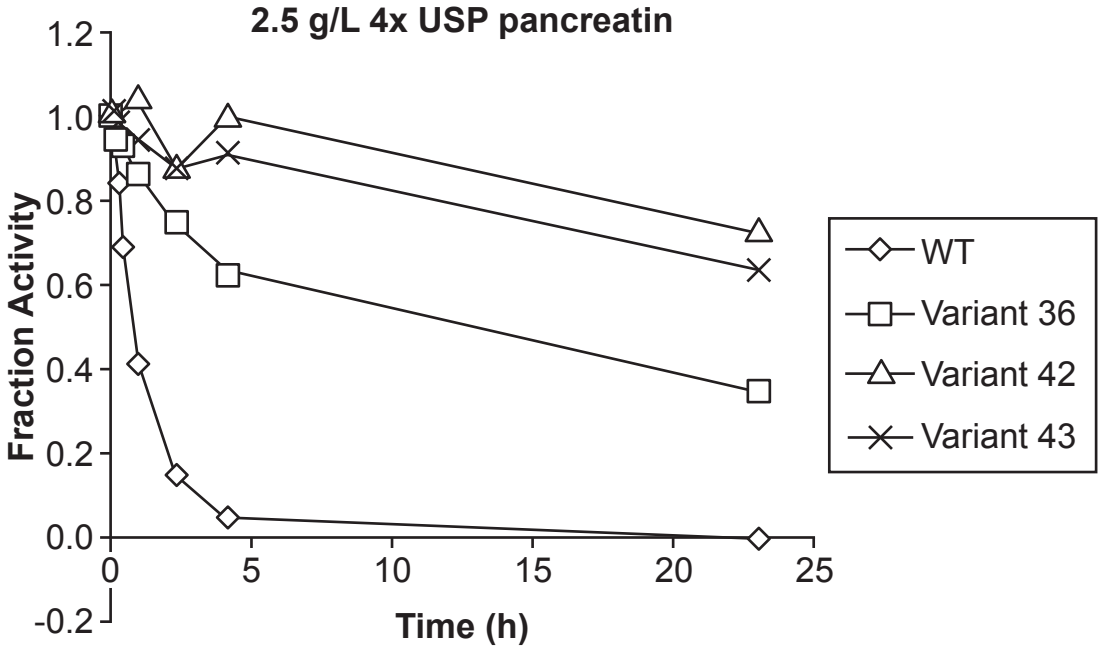

2.5 g/L 4x USP pancreatin
Fraction Activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
5
10
15
20
25
Time (h)
WT
Variant 36
Variant 42
Variant 43

FIG. 6

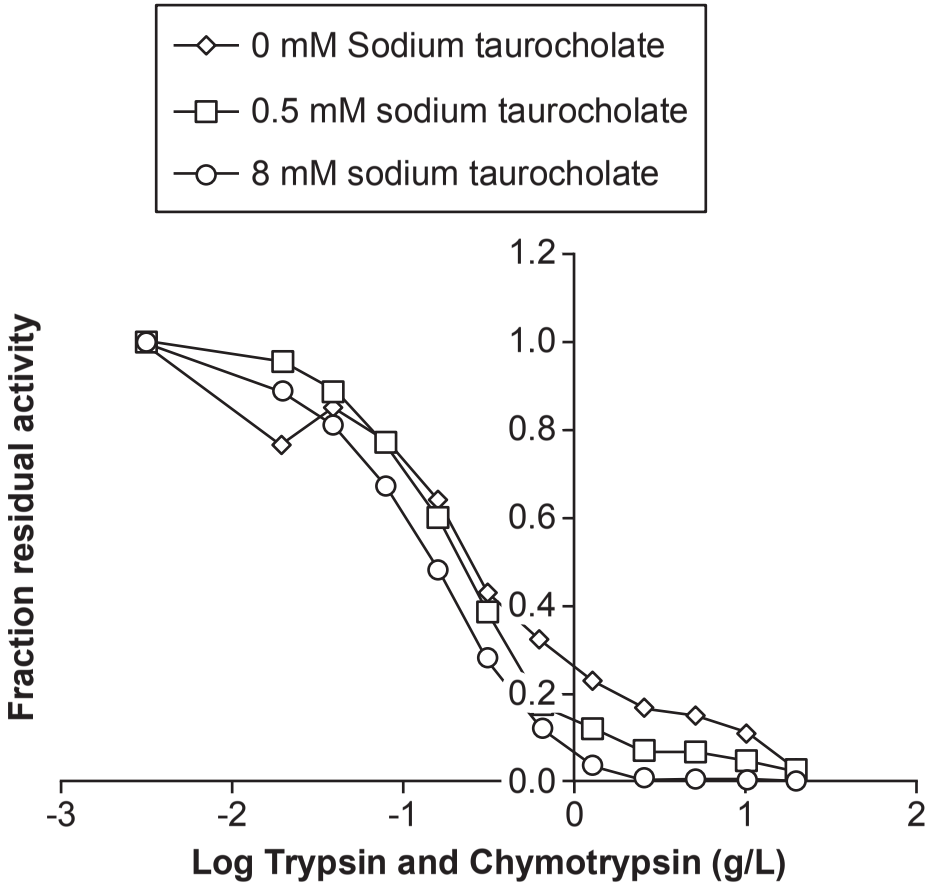

0 mM Sodium taurocholate
0.5 mM sodium taurocholate
8 mM sodium taurocholate
Fraction residual activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-3
-2
-1
0
1
2
Log Trypsin and Chymotrypsin (g/L)

FIG. 7

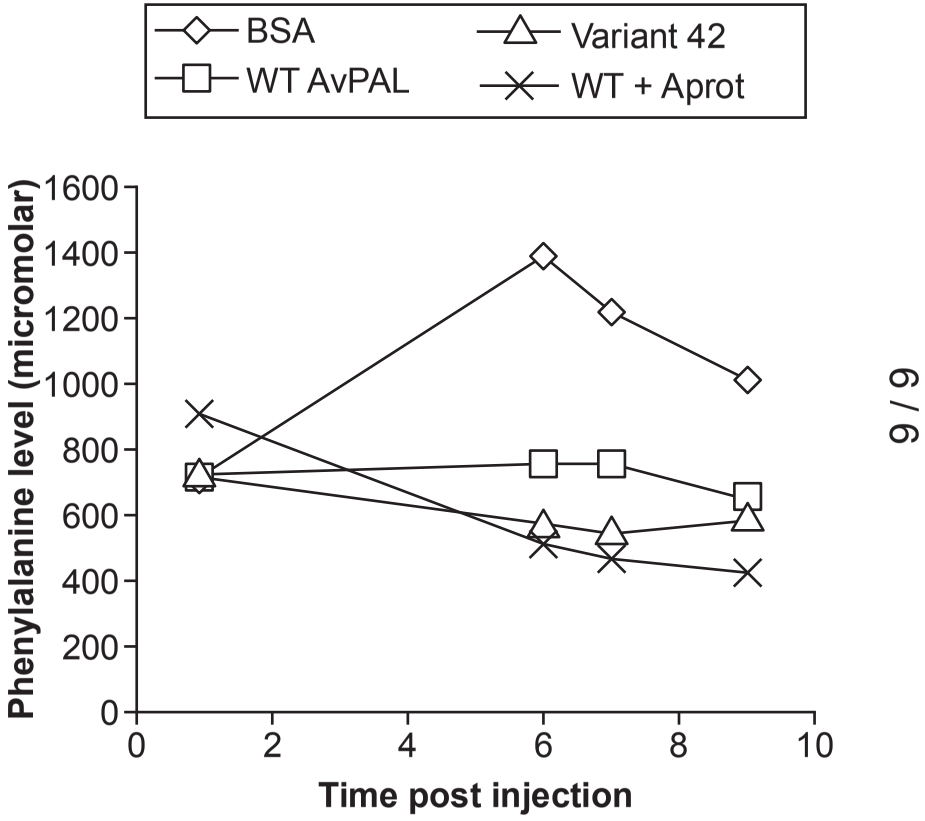

BSA
Variant 42
WT AvPAL
WT + Aprot
Phenylalanine level (micromolar)
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
Time post injection

FIG. 8